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ISOLATION AND STRUCTURAL STUDIES
ON A LOCUST FEEDING INHIBITOR
FROM NEEM SEEDS

by

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A thesis submitted to the University of Keele in
partial fulfilment of the requirements for the
Degree of Doctor of Philosophy

SUMMARY

Two species of Meliaceae, neem (*Azadirachta indica* A.Juss) and *Melia azedarach* L. have been reported by various workers to inhibit feeding in the desert locust (*Schistocerca gregaria*). In this work a systematic investigation of extracts of neem seeds has been carried out in order to isolate a locust feeding inhibitor. Antifeeding activity was measured by presenting fifth instar hoppers of the desert locust with filter papers which had been impregnated with a solution of the extract to be tested and allowed to dry and then sprayed with 0.25 M. sucrose solution and allowed to dry again. In this way a locust feeding inhibitor, azadirachtin, has been isolated. This compound completely inhibits feeding with test solutions down to a concentration of 40 µg./l.

Azadirachtin, molecular formula $C_{35}H_{44}O_{16}$, is a colourless non-crystalline compound which is probably related to the limonoids and triterpenoids already isolated from Meliaceae, but is more highly oxygenated. It has so far been shown to contain two hydroxyl groups, two acetoxy groups, one tiglate ester group, two methyl ester groups and a dihydrofuran ring.

ACKNOWLEDGEMENTS

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INTRODUCTION

Azadirachta indica A. Juss of the family Meliaceae is commonly known as the neem or nim tree and is found in India and parts of Africa. The neem tree, a large evergreen tree which often attains 40 to 50 ft. in height, has been widely cultivated because of its reputed medicinal properties.¹ The bark, leaves and fruits have been used from ancient times in Hindu medicine; the bark and leaves are regarded as useful in the treatment of fever, thirst, nausea and skin diseases; the fruits are reputed to act as a purgative, emollient and anthelmintic. Neem oil is used by the villagers in India as a hair tonic and is reputed to cure rheumatism, scrofulous glands, leprosy and a variety of skin infections. Caius and Mhoskar² in 1923 investigated the reputed anthelmintic properties of the juice of neem leaves and neem oil, they concluded that both treatments were quite ineffective.

Another species of the Meliaceae, *Melia azedarach* L. which is cultivated as an ornamental shade tree in India is reputed to possess similar therapeutic properties to those of neem.³ There is apparently a certain amount of confusion between these two species of Meliaceae in the literature, because *Azadirachta indica* is also known as *Melia azadirachta* or *Melia indica*.

Various parts of neem and *Melia azedarach* L. have long been known to inhibit feeding in the desert locust (*Schistocerca gregaria*).

In 1937 Volkonsky⁴ reported that extracts of the leaves of *Melia azedarach* L. when sprinkled onto the leaves of other plants gave protection against the locust, since forcibly introducing leaves into the insects produced no effect he concluded that it is the taste which is repulsive to the locust. Chauvin in 1946⁵ claimed to have isolated from the leaves of *Melia azedarach* L. a substance which he called meliatin, by extraction with chloroform, precipitation with light petroleum and chromatography. Because meliatin was eluted as a yellow band in the chromatography Chauvin said it was "carotenoid like". Chauvin tested for the repellency or anti-feeding activity by spraying aqueous suspensions of the extracts onto leaves which would normally be eaten by the locust.

In 1950 Krishnamurti and Rao⁶ reported that neem leaves showed insect repellency in stored grain. Sinha and Gulati⁷ extracted the seed cake left after oil expression of neem seeds with alcohol either by Soxhlet extraction or by percolation at room temperature. The former extract showed repellent action against the migratory locust (*Locusta migratoria*) and the latter relatively greater repellency to the migratory locust and the desert locust.

Mrs. Shpan-Gabrielith^{8,9} working at the Anti-Locust Research Centre London in 1964-1965 tested several plants for locust repellent activity amongst them *Melia azedarach* L. which was found to be repellent. The method adopted to test extracts of plants was to impregnate filter papers with a solution of the extract allow them to dry and then spray them with 0.25M sucrose solutions and allow them to dry again, these

papers were presented to groups of fifth instar hoppers of the desert locust which had been starved for 24 hours previously. In the case of paper chromatograms, these were sprayed with 0.25M sucrose solution, allowed to dry and then the chromatograms were cut into strips and presented to the groups of insects. When the ethanol extract of *Melia azedarach* L. was chromatographed on a column of silica the repellent activity was found to be eluted by ether and methanol. On ascending paper chromatography eluting with toluene, the activity was found to remain at the origin whereas on eluting with ethanol-water (170:50) the activity was found at the top of the chromatogram.

An examination of the extracts of the seeds of *Melia azedarach* L. was carried out by extracting the seeds in turn with water, methanol and light petroleum; the water extract was found to be most repellent and the light petroleum extract least repellent.

It was clear from the results of Shpan-Gabrielith that a more detailed chemical investigation was necessary in order to isolate the component or components responsible for the anti-feeding activity.

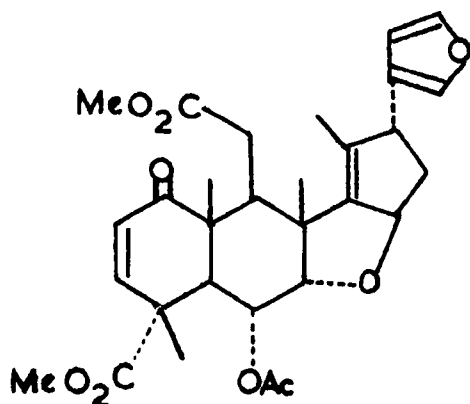
Because of the reputed therapeutic properties of neem and *Melia azedarach* L. the investigation of their chemical constituents has attracted the attention of several groups of workers over the last 50 years. Early workers examined the fatty acids from neem oil. Chatterjee¹⁰ claimed to have isolated a new acid, margosic acid, but this was later shown by Roy and Dutt¹¹ to be a mixture of well known fatty acids with some bitter impurities. Several groups of Indian workers^{12,13,14} between

1920 and 1940 claimed to have isolated bitter principles from neem, but there is no evidence that these were single compounds.

The introduction of the modern techniques of structure elucidation, especially nuclear magnetic resonance spectroscopy, has led to considerable progress being made during the last five years in the complete identification of some of the constituents.

Several tetranortriterpenoids have been isolated from neem; these are limonoids, a class of C_{26} degraded triterpenes which are believed to arise biogenetically from tetracyclic triterpenes and which all have a β -substituted furan ring.

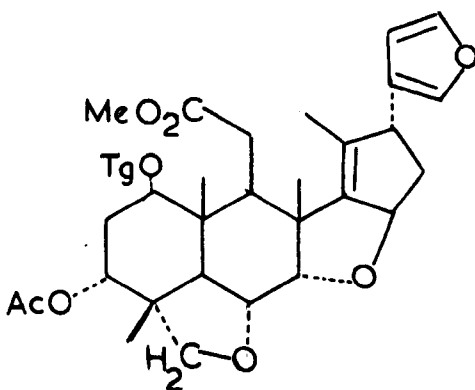
Nimbin (1) $C_{30}H_{36}O_9$ was first isolated from neem oil by Siddiqui¹⁵ in 1942, its complete structure and stereochemistry was reported by Narayanan *et al.*¹⁶ in 1964 and this was later confirmed by Overton *et al.*¹⁷



(1)

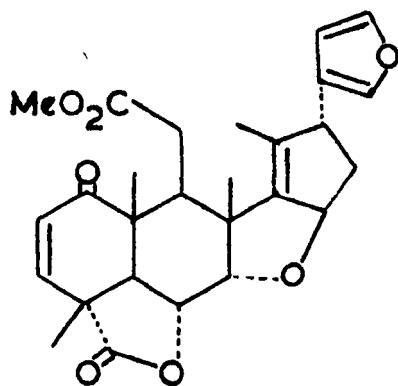
Deacetyl nimbin has been isolated along with nimbin from the seeds and bark of neem by Narayanan and Iyer.¹⁸

Salannin (2) $C_{34}H_{44}O_9$, was first isolated from neem oil in 1964 by Overton¹⁹ *et al.* who in 1968 reported²⁰ its complete structure.



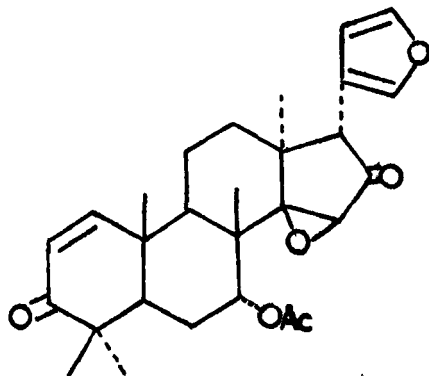
(2) Tg = Tigloyl

The isolation and structure determination of nimbolide (3) $C_{27}H_{30}O_7$ from neem leaves was reported by Ekong²¹ in 1967.

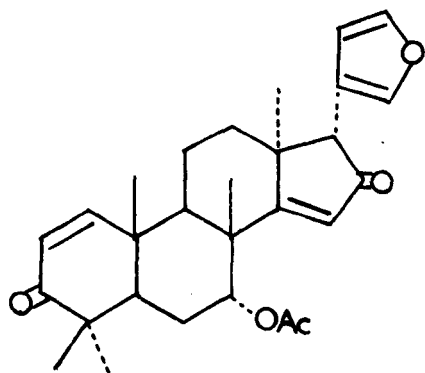


(3)

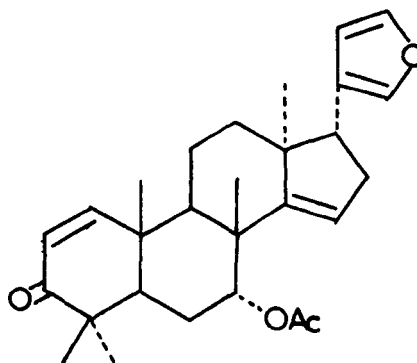
Lavie and Jain²² in 1967 reported the isolation from neem oil of three new tetranortriterpenoids epoxyazadiradione (4), azadiradione (5) and azadirone (6) along with gedunin (7) and 7-deacetyl gedunin (8) which had previously been isolated from other species of the Meliaceae.



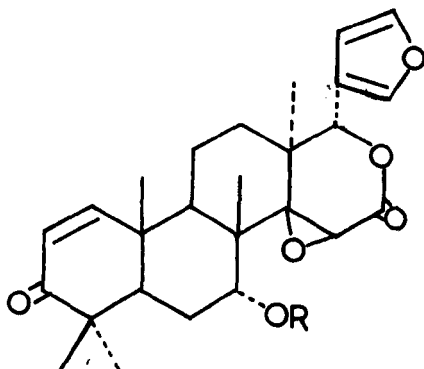
(4)



(5)



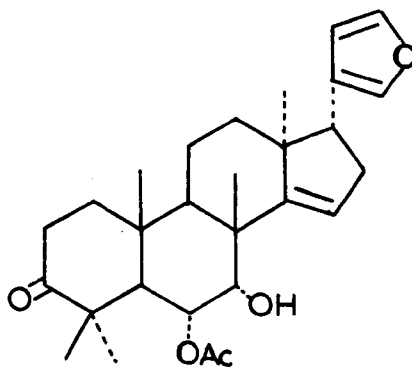
(6)



(7) R = Ac

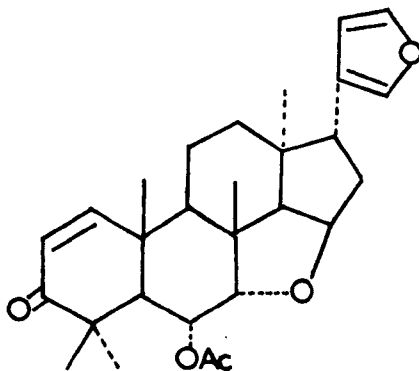
(8) R = H

The isolation of meldenin²³ (9) $C_{28}H_{38}O_5$ from neem oil was reported in 1968 by McCrindle *et al.* who also reported the isolation of nimbinin which was first described by Siddiqui.¹⁵ The structure of nimbinin had been partially elucidated by Narayanan *et al.*²⁴ in 1967, McCrindle and his colleagues concluded that nimbinin may be identical with epoxyazadiradione (4) although there is a discrepancy in the recorded specific rotations.



(9)

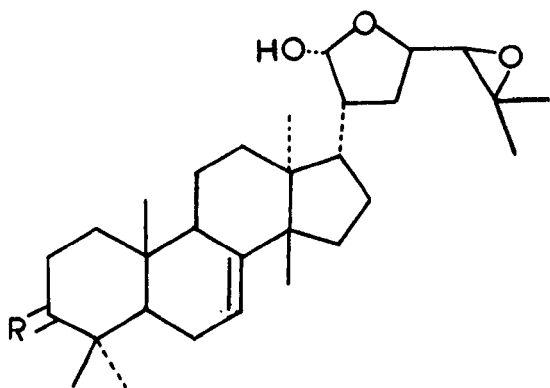
Another limonoid vepinin (10) has recently been isolated from neem oil by Narayanan *et al.*²⁵



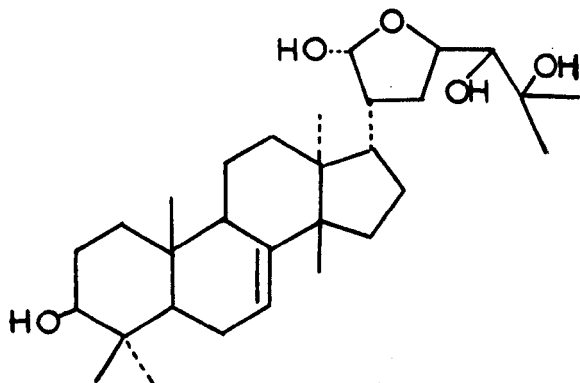
(10)

Several tetracyclic triterpenes with the stereochemistry of tirucallane at C-13, C-14 and C-17 have been isolated from neem and *Melia azedarach* L. Melianone (11) and melianol (12) were isolated from the seeds of *Melia azedarach* L. by Lavie *et al.*^{26,27} in 1966.

While the work described in this thesis was in progress the same group²⁸ isolated from the same source and also from neem oil, meliantriol (13), a triterpene which is claimed to possess locust anti-feeding activity.



(11) R = O

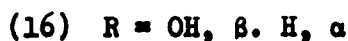
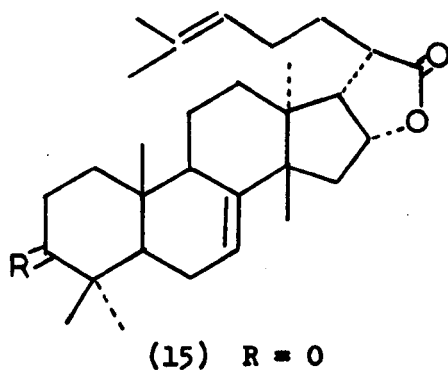
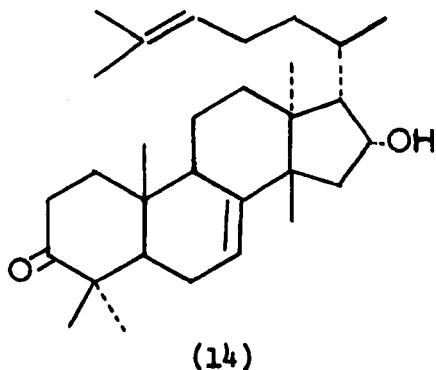


(13)

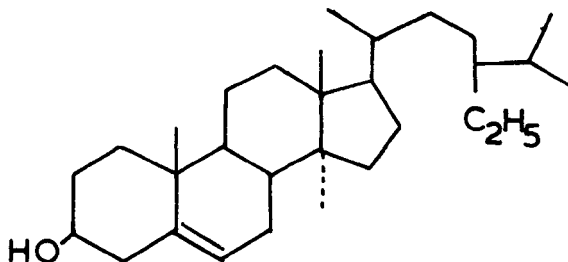
(12) R = OH, β . H, α

Terpenoids of the melianone, melianol and meliantriol type are believed to be the biogenetic precursors of the limonoids. Degradation of the side chain with the loss of four carbon atoms could give rise to the β -substituted furan ring of the limonoids, a mechanism for this reaction has been suggested by Bevan and Halsall.²⁹

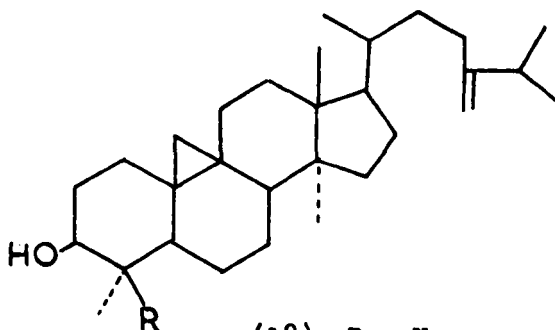
The formation of a furan ring coupled with a shift of the C-14 methyl to C-8 would give rise to the apo-euphol type structure that is found in meldenin (9) and azadirone (6). Further oxidation would give azadiradione (5) and epoxyazadiradione (4) which on Baeyer-Villiger type oxidation of the ketone would give the lactone structure of gedunin (7). Alternatively closure of the C-7 oxygen to C-15 would give rise to limonoids with a vepinin (10) type structure, further modification including cleavage of ring C would give rise to limonoids such as nimbin (1), salannin (2) and nimbolide (3). In this way the triterpenoids and limonoids isolated from neem and *Melia azedarach* L. fit into a scheme of increasing oxygenation. Kulinone (14), kulactone (15) and kulolactone (16) isolated from the leaves of *Melia azedarach* L. by Chang and Chiang^{30,31} may represent an earlier stage in the sequence of oxidation. These authors suggest, since these compounds are oxygenated at C-16, that oxygenation at C-16 may occur prior to degradation of the side chain to give the tetranortriterpenoids which are oxygenated at C-16.



Ekong *et al.*³² have recently isolated from the wood oil of the neem tree three previously known tetracyclic triterpenoids, β -sitosterol (17), cycloeucalenol (18) and 24-methylene-cycloartenol (19), which belong to the lanostane group in respect of their stereochemistry at C-13, C-14 and C-17.



(17)



(18) R = H

(19) R = Me

Ekong suggested that these triterpenoids represent members of another oxidation series parallel to the one described above.

DISCUSSION

Isolation of Azadirachtin

The object of this work was to carry out an investigation of the property of neem to inhibit feeding in the desert locust (*Schistocerca gregaria*), with the ultimate aim of isolating the component or components responsible for this antifeeding activity. Neem seeds were available from India through the kind co-operation of the Tropical Products Institute and hence provided a convenient source of plant material for this work.

The method used to test fractions for antifeeding activity was that used by Shpan-Gabrielith^{8,9} in the work on *Melia azedarach* L. (see Introduction). Filter papers impregnated with the fractions to be tested and with sucrose were presented to groups of mid-fifth instar hoppers of the desert locust which had been starved for 24 hours. Because of the more limited supply of insects, groups of four were used rather than groups of ten which had been used by Shpan-Gabrielith. Each time a feeding test was carried out one group of insects was given filter papers impregnated with sucrose only to act as a control. In several of the fractions tested it was found that appreciably more of the test paper was eaten than sucrose control paper, this may be attributable to the presence of positive feeding stimuli in some of the fractions.

The term repellent has been widely used to describe the property of neem and *Melia azedarach* L. to inhibit feeding in the locust. Observation of the feeding tests carried out in this work do not confirm this as a strictly accurate description. The insects were not repelled away from any of the papers impregnated with extracts of neem, the activity that was observed would seem to be only a gustatory one, because the insects examined all the papers in the same way with their mouth parts. The terms feeding inhibition, feeding suppression and antifeeding activity provide a more accurate description of the phenomenon. Fractions were considered to possess antifeeding activity if the test papers which were impregnated with them were not eaten.

The total amount of sucrose control paper eaten during a period of eight hours by four mid-fifth instar hoppers varied between 100 and 300 mg. The amounts of paper eaten during the winter months, when the grass on which the locusts were fed prior to testing was senescent, tended to be lower than in the summer months when the locusts were being fed on fresh grass.

The neem seed consists of a brittle shell surrounding a soft kernel. In the initial experiment kernels of a sample of Nigerian neem seeds were separated from the shells and extracted in turn with water, 95% ethanol and finally light petroleum, at the concentrations at which the solutions were tested for antifeeding activity, which were equivalent to 350g. and 35g. of kernels in a litre, all were found

to possess activity. The shells were extracted in a Soxhlet apparatus with 95% ethanol, the resulting solution was also found to have anti-feeding activity.

Now, having ascertained that extracts of the seeds possessed antifeeding activity, an experiment was carried out to determine whether this was due to volatile or involatile components. Kernels of neem seeds, this time from India, were ground with water, the solution obtained was distilled to give a residue and distillate. The residue of the seeds was then extracted in a Soxhlet apparatus by 95% ethanol followed by light petroleum, the resulting solutions were evaporated to give the residues and distillates. All the residues and distillates were tested for antifeeding activity at concentrations equivalent to 5g., 3.3g. and 1g. of kernels in a litre. All the distillates were found to be inactive at these concentrations, the light petroleum extract only showed activity at the two higher concentrations, however the aqueous and ethanol residues showed activity at all three concentrations. This experiment showed that the antifeeding component is an involatile substance and also confirmed that neem seeds from India as well as from Nigeria possessed antifeeding activity.

Kernels of neem seeds were extracted by grinding and then percolating with 95% ethanol followed by grinding with water. The combined weight of the ethanol and aqueous residues was similar to that of the ethanol and aqueous residues which had been obtained in the previous extraction when the extractions had been carried out in the reverse order.

In each case the extraction carried out first gave the larger residue, therefore there must have been a certain amount of material which was soluble in both water and ethanol. Since the total weights obtained in each extraction were very similar there seemed to be no advantage in using the more time consuming method of percolation to extract the seeds.

Active material was soluble in both water and ethanol, however the aqueous extraction was more difficult to carry out because the solution did not filter easily, the filter paper tended to become blocked and difficulty was encountered with foaming during the evaporation. For this reason further work on the isolation of an anti-feeding component was carried out on ethanol extracts. The aqueous extract was not further examined but in view of the nature of the antifeeding component later isolated from the ethanol extract it is probable that the activity of the aqueous extract was due to this same component.

The ethanol extract was an oily residue containing solid material, in attempt to separate this extract into fractions of different polarity, solvent partition was tried. Partition of the ethanol extract between chloroform and water and between n-butanol and water was unsuccessful resulting only in the formation of emulsions. However partition between light petroleum and methanol with 5% added water was successful. The fractions isolated from the two phases were tested for antifeeding activity at concentrations equivalent to lg. seeds/l 0.66g. seeds/l and 0.5g. seeds/l. The fraction from the methanol phase

was active at all three concentrations, that from the light petroleum phase showed activity only at the highest concentration. Apparently an effective separation had been achieved, a possible improvement which was adopted in later isolations was to wash the light petroleum layer with a further quantity of aqueous methanol (5:95).

In an attempt to achieve further separation, the residue from the methanol phase of the partition was subjected to chromatography, and since the indication from the earlier extractions was that the active material was fairly polar, this was carried out on a column of activity III alumina. The residue was put onto the top of the column as a powder and the column was eluted successively with solvent mixtures of increasing polarity, beginning with light petroleum in which the residue appeared to be insoluble. Fractions were collected as shown in the table (see Experimental), groups of fractions were tested for anti-feeding activity. The first active group was that eluted by solvent mixtures ranging from benzene-ether (9:1) to ether, the last active group of fractions was eluted by methanol. The grouping of fractions together for testing was necessary to keep the number of tests down to manageable proportions. That solvent mixtures of such high polarity are required to elute the active material from an activity III alumina column is further indication that this material is of fairly high polarity. The active material may have been spread over so many fractions because the crude material was put onto the column as a solid and perhaps did not dissolve smoothly as the polarity of the solvent was increased.

All further work on the isolation of an antifeeding component was carried out on the residue from the methanol phase of a partition of the ethanol extract of neem between aqueous methanol and light petroleum. The ethanol extract, obtained by grinding the seeds in a Waring Blendor, was partitioned between aqueous methanol (5:95) and light petroleum, the light petroleum was washed with a further quantity of aqueous methanol, (5:95) and the combined methanol extracts were evaporated leaving a brown sticky residue, which on pumping at 0.1 mm. Hg. could be obtained as a brittle solid.

The chromatography of the methanol partition residue on alumina (activity III) was repeated. To avoid putting the material on the column as a solid it was decided to first absorb the material onto alumina. A methanol solution of the residue was mixed with alumina and then evaporated, a lumpy residue was obtained, silica gel and more methanol were added and the mixture was again evaporated, this time the residue was a free flowing powder. This powder was packed onto the top of the alumina (activity III) column which was eluted with benzene and benzene-methanol mixtures. All the activity was found in the benzene-methanol (9:1) fraction, this contained about 30% of the material put onto the column. The active fraction was further chromatographed on another alumina (activity III) column, this time eluting more carefully with benzene, benzene-ether, ether and ether-methanol mixtures. Tests for antifeeding activity were carried out on the fractions at increasing dilution, activity was first found to be eluted by benzene-ether (3:2) but was found to be mainly in the ether-methanol (9:1) fraction.

The alumina chromatography of the methanol partition residue was repeated on a larger scale, a portion of the fraction eluted exhaustively by ether-methanol (9:1) was further chromatographed on another alumina column (activity III), the activity was found to be mainly in the ether-methanol (9:1) and ether-methanol (4:1) fractions, confirming the results obtained with the small scale chromatography.

Crystallization of the benzene fraction from 95% ethanol gave a colourless microcrystalline solid which showed in its infrared spectrum an intense sharp absorption at 875 cm^{-1} characteristic of the β -substituted furan ring of the tetranortriterpenoids, and analysed for $\text{C}_{30}\text{H}_{36}\text{O}_9$, the molecular formula of nimbin.^{16,17} This material was shown by infrared spectroscopy and thin layer chromatography to be identical with a sample of nimbin obtained from Dr. N.S. Narasimhan. Since the benzene fraction from which nimbin was isolated showed no activity, then nimbin must be inactive.

The active ether-methanol (9:1) fraction was further chromatographed on a column of Florisil, eluting with benzene, benzene-ether mixtures, ether and ethyl acetate mixtures. Feeding tests showed that all the ether-ethyl acetate fractions possessed activity, these fractions were combined together and subjected to preparative layer chromatography; three components were isolated in poor yield. Each of these components was tested for antifeeding activity at increasing dilution. The least polar component was found to be the most active showing activity at 0.2 mg/l., the component from the middle band showed activity at 2 mg/l. and the lower band did not show any activity at the

concentration at which it was tested.

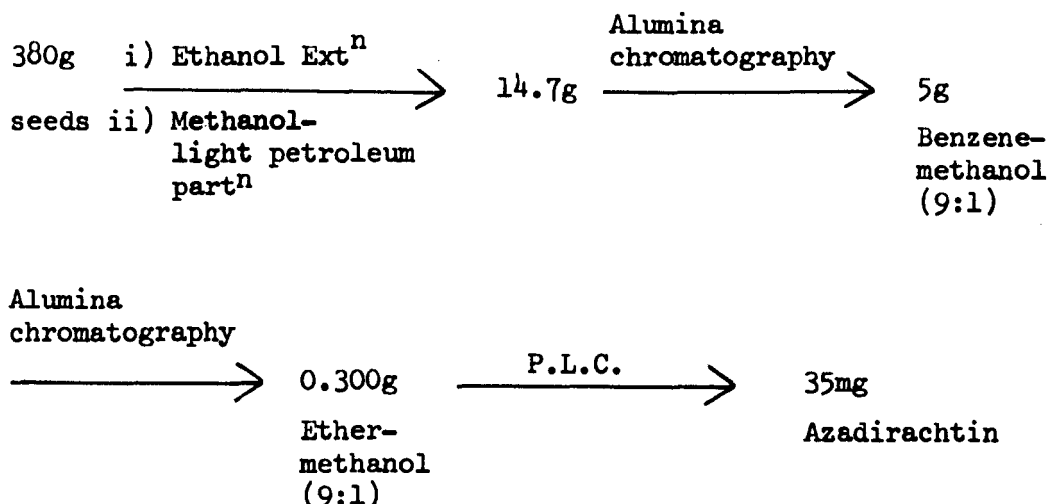
Alumina chromatography of the active benzene-methanol (9:1) fraction was repeated on a larger scale, in order to isolate the active components in greater amounts. The fractions eluted by ether, ether-methanol (9:1) and ether methanol (4:1) showed on thin layer chromatography components with R_F values in the region of the upper band of active material which had been isolated previously, the ether-methanol (9:1) fraction appeared to contain this component in the greatest proportion. The three fractions were tested for anti-feeding activity and were all found to be active, thus confirming that R_F values on thin layer chromatography could be correlated with the antifeeding activity.

Two components were separated by preparative layer chromatography of the ether-methanol (9:1) fraction, these corresponded in R_F to the upper and middle bands of active material which had been isolated previously. However although the upper component behaved as a single substance, the more polar material appeared to contain some of the upper component as impurity when the fractions were subjected to thin layer chromatography.

The two components were tested for antifeeding activity, the upper component at 0.4 mg/l. and 0.2 mg/l. and the more polar component at 2.4 mg/l. and 1.2 mg/l. at these concentrations both the fractions showed activity. It is probable that the activity of the more polar component was due to the presence of the upper component as impurity. All future attention was concentrated on the highly active upper

component, which was given the name azadirachtin.

The scheme of separation used to isolate azadirachtin is summarised below



At this stage it was necessary to try to improve the scheme of isolation so that azadirachtin could be obtained in sufficient quantities for chemical studies. It was considered desirable to carry out some more simple preliminary separations in order that the scale of the chromatography could be reduced. Examination of the methanol partition residue on thin layer chromatography showed that it contained a great amount of material very much more polar than azadirachtin and also a considerable amount of less polar material.

In order to try to separate out some of the more polar material a partition was carried out between methylene chloride and water, the water layer, which became emulsified, gave a residue which on thin layer chromatography showed only polar material, with no

components with R_F in the region of that of azadirachtin.

The methylene chloride was evaporated to smaller volume and then poured into light petroleum, a precipitate was formed which was filtered off. Thin layer chromatography of the residue from the light petroleum showed that separation of the less polar material had been achieved because this showed only components with R_F greater than that of azadirachtin. Therefore by the partition and precipitation some of the components more polar and less polar than azadirachtin had been separated out, this represented about 50% of the methanol partition residue.

A portion of the precipitated solid was subjected to column chromatography on alumina but this time, for some inexplicable reason, no efficient separation was achieved, all the fractions which were eluted by solvent mixtures ranging in polarity from benzene to ether-acetone were found by thin layer chromatography to contain components with R_F in the region of that of azadirachtin. These fractions were combined, their total weight was about 40% of the weight of the material put onto the column, therefore a considerable amount of more polar material had been separated out.

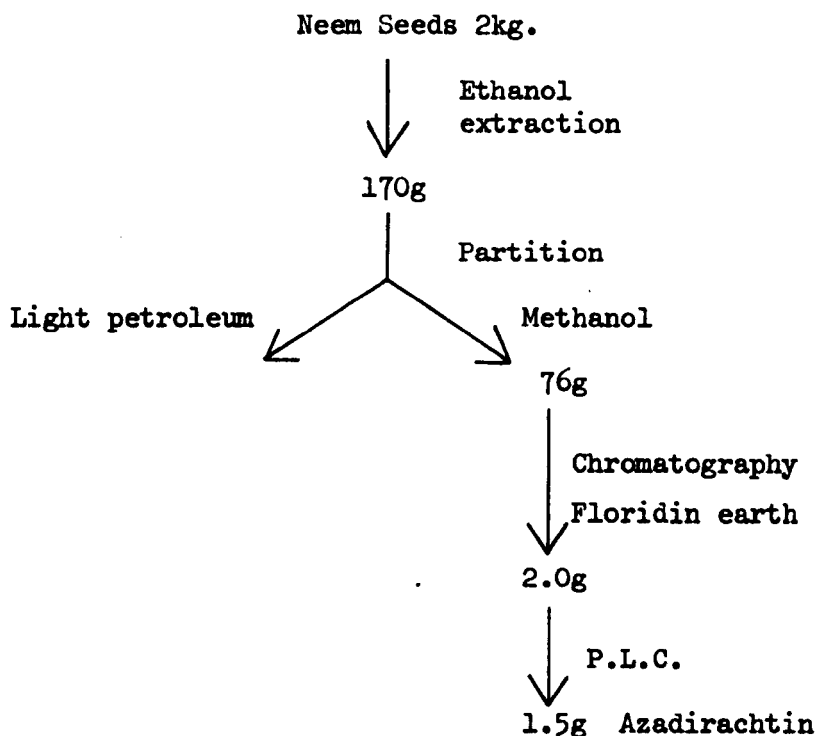
The combined fractions were subjected to chromatography on a column of BDH silica gel, this proved to be very much less active than alumina (activity III), because all the material with R_F on thin layer chromatography in the region of that of azadirachtin, was eluted by benzene whereas with the alumina column it was eluted by ether-methanol. The benzene fraction, which represented about 60% of the material put onto

the column, was subjected to chromatography on a column of Florisil, this showed higher activity than the BDH silica gel, azadirachtin being eluted over a large number of fractions by benzene-ether mixtures, the fractions which contained azadirachtin in the greatest proportion were combined and subjected to preparative layer chromatography.

Azadirachtin was isolated along with a smaller amount of a component which had an R_F slightly greater than that of azadirachtin. The two components were tested for antifeeding activity, the azadirachtin was found to be active down to a concentration of at least 0.07 mg/l., the other component showed no activity at 3.5 mg/l. and 0.35 mg/l.

This procedure was still rather long and involved, larger scale isolations involved the use of large amounts of Florisil, which is expensive, and large volumes of solvent, so further efforts were made to try to improve the isolation. The methanol partition residue was chromatographed directly on a column of Floridin earth made up in toluene, eluting straight away with ether acetone (95:5). The components less polar than azadirachtin were eluted quickly from the column followed by azadirachtin which was eluted from the column contaminated with the two components of R_F slightly greater and slightly less which had been observed previously. All the fractions which were found to contain an appreciable amount of azadirachtin by n.m.r. spectroscopy and thin layer chromatography were combined and subjected to preparative layer chromatography.

This separation is summarised below.



Azadirachtin was isolated as a colourless glassy material, insoluble in ether and light petroleum, slightly soluble in carbon tetrachloride, soluble in benzene and toluene and very soluble in ethanol, methanol, acetone and chloroform. Attempts to crystallize azadirachtin were not very successful, the only solvent from which it would crystallize at all was carbon tetrachloride and then only as a fine powder.

Feeding tests were carried out to find the limiting activity of azadirachtin. Three separate tests were carried out on different

batches of insects. In all cases papers impregnated with a solution of azadirachtin at a concentration of 0.08 mg./l. were not eaten, in two of the tests a solution concentration of 0.04 mg./l. was used and it was found that in one case the test papers were eaten and in the other case they were not. From this it would appear that a test solution concentration of 0.04 mg./l. is near the limit of activity of azadirachtin.

While this work was in progress Lavie *et al.*²⁸ reported the isolation of a locust feeding inhibitor, meliantriol, from *Melia azedarach* L. and neem oil which they showed to have a triterpene structure (13). These workers also used the filter paper method to test fractions for antifeeding activity but quoted their results as a weight of extract per cm^2 of filter paper which produced 100% anti-feeding activity, so that in order to compare the activities of azadirachtin and meliantriol it is necessary to convert a concentration of test solution into a weight of extract per cm^2 of filter paper. Assuming that one 5.5 cm filter paper absorbs about 0.5 ml. of test solution, a concentration of 0.04 mg./l. is equivalent to approximately 1 ng./cm^2 , compared to the quoted limiting activity of meliantriol of $8 \text{ } \mu\text{g./cm}^2$. However a crude ethanol extract of neem seeds using the same conversion would be active at $1 \text{ } \mu\text{g./cm}^2$, that is a higher activity than that quoted for meliantriol.

The systematic separation and testing of fractions by Lavie *et al.* was apparently carried out on *Melia azedarach* L. and not on neem oil, no indication is given on the relative activities of crude extracts of the

two species. There is the possibility that although meliantriol was shown to be present in both species, azadirachtin may only be present in neem, and hence although both extracts show some activity, the neem extract is far more active. Azadirachtin, it will be seen later, does not bear any close resemblance to meliantriol.

Structural Studies on Azadirachtin

The infrared spectrum of azadirachtin showed a broad absorption at 3460 cm^{-1} which was assigned to the presence of at least one hydroxyl group, an intense absorption from 1700 to 1740 cm^{-1} which indicated the presence of a number of carbonyl groups, absorptions at 1625 cm^{-1} and 1655 cm^{-1} suggested the presence of C=C bonds.

The ultraviolet spectrum of azadirachtin showed only one absorption at $\lambda_{\text{max.}} 217\text{ nm}$, hence azadirachtin contains no aromatic rings. This absorption was shown not to be due to $\alpha\beta$ -unsaturated ketone or aldehyde groups because on addition of sodium borohydride to an ethanol solution there was no decrease in the intensity of this absorption, which would occur on reduction of these groups.

The n.m.r. spectrum of azadirachtin at 60 MHz and 100 MHz is shown in figs. 1 and 2. Taking the integral of the smallest absorptions e.g. at $\tau 3.55$, $\tau 4.35$ and $\tau 4.5$ as representing one proton then the total integral was somewhere between 40 and 50 protons. On shaking with D_2O a one proton absorption disappeared, the position of this absorption was between $\tau 6.8$ and $\tau 7.0$ depending upon concentration. In the n.m.r.

60MHz. N.M.R. Spectrum of Azadirachtin

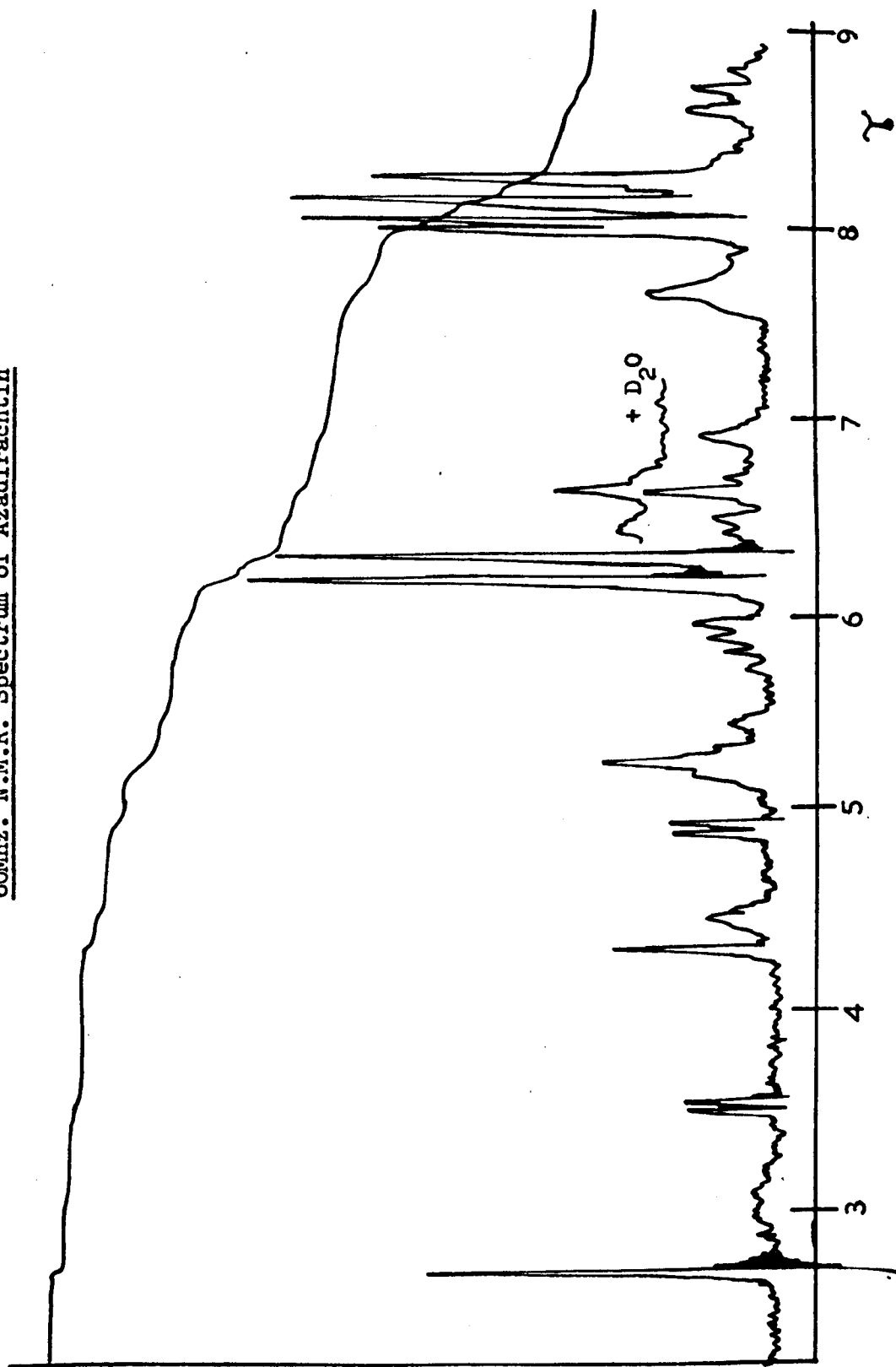


Figure 1

100MHz. N.M.R. Spectrum of Azadirachtin

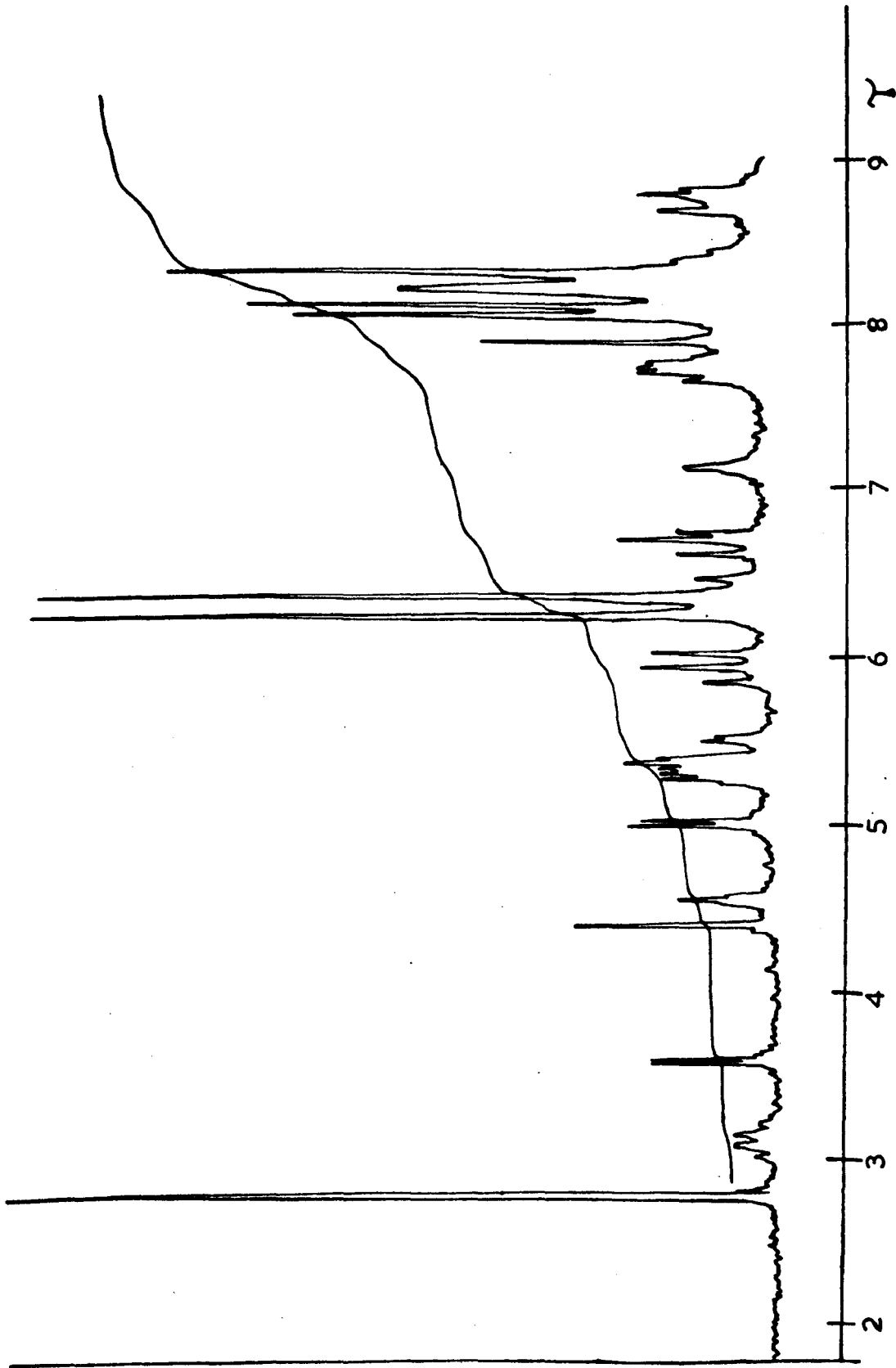


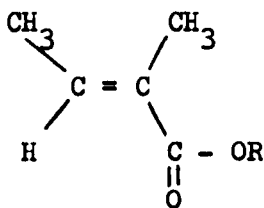
Figure 2

spectra of some samples of azadirachtin there was a sharp absorption which varied in position between $\tau 4.9$ and $\tau 5.0$, this also exchanged with D_2O . The two three-proton singlets at $\tau 6.25$ and $\tau 6.35$ can be assigned to the methyl protons of two carbomethoxy groups, the intense absorption in the carbonyl region of the infrared spectrum, mass spectral data and hydrolysis experiments confirm this assignment.

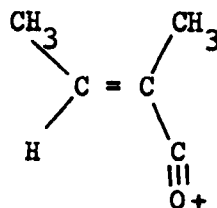
The two three-proton singlets at $\tau 8.0$ and $\tau 8.05$ can be assigned to the protons of methyl groups next to carbonyl, these absorptions are at rather high field to be due to methyl ketone groups and so must be due to the protons of two acetate groups, the intense absorption in the infrared spectrum at 1740 cm^{-1} and the loss of 60 mass units due to loss of acetic acid in the mass spectrum are further evidence for this assignment.

The absorption at $\tau 8.7$ varied in intensity with different samples of azadirachtin some of this was probably due to hydrocarbon grease impurity from the silica used in preparative layer chromatography. The n.m.r. spectrum of a sample of azadirachtin recrystallized from carbon tetrachloride showed a reduction in intensity of this absorption, but some absorption was still visible in this position.

There was a strong ion in the mass spectrum of azadirachtin at m/e 83 corresponding to an ion $C_5H_7O^+$. The tiglate ester group (20), which has been found in a number of naturally occurring compounds including salannin^{19,20} (2) a tetranortriterpenoid isolated from neem, would give rise to such an ion (21).



(20)



(21)

The n.m.r. spectrum of azadirachtin showed a broad multiplet at τ 3.0 and absorptions at τ 8.15 and τ 8.25 which are characteristic of the tiglate group, as also is the absorption at 217 nm. in the ultra-violet spectrum. The infrared spectrum of methyl tiglate showed a carbonyl absorption at $1,710 \text{ cm}^{-1}$ and a C=C stretching band at $1,650 \text{ cm}^{-1}$, corresponding absorptions appeared in the spectrum of azadirachtin.

The presence of a tiglate ester group was confirmed by alkaline hydrolysis. Azadirachtin was hydrolysed under vigorous alkaline conditions. The resulting mixture was acidified and extracted into ethereal diazomethane and the ether solution was subjected to gas chromatography. The gas chromatogram showed a peak corresponding in retention time to that of methyl tiglate. It was not possible to identify methyl acetate formed from the acetic acid produced in the hydrolysis because the diazomethane was prepared from N-nitrosomethyl urea which contained acetic acid as impurity.

The possibility that the ester group in azadirachtin was an angelate ester and that isomerisation to tiglate had taken place on hydrolysis can be ruled out by n.m.r. data. Fraser³³ reported the

chemical shifts of the protons in methyl tiglate and methyl angelate:

	Methyl Tiglate	Methyl Angelate
α -CH ₃	τ 8.20	τ 8.13
β -CH ₃	τ 8.25	τ 8.05
β -H	τ 3.28	τ 4.03

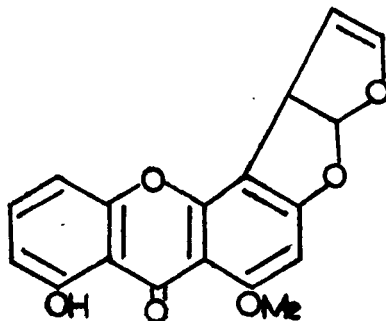
In the spectrum of azadirachtin the multiplet at τ 3.0 corresponds more closely to the position of absorption of the β -H proton of methyl tiglate.

The 60 MHz n.m.r. spectrum of methyl tiglate showed the α -CH₃ singlet superimposed over the low field component of the β -CH₃ doublet, these absorptions were not further split; whereas the spectrum of methyl angelate showed the α -CH₃ singlet superimposed over the high field component of the β -CH₃ doublet and in this case the absorptions were further split because the coupling between the methyl protons was greater than in the case of methyl tiglate. The appearance of the absorptions at τ 8.20 and τ 8.28 suggested the presence of an absorption due to a tiglate ester with a two or three proton singlet superimposed on the high field component of the β -CH₃ doublet. This was later confirmed by spin decoupling, irradiating at τ 3.0 the β -CH₃ doublet collapsed to a singlet revealing a singlet at τ 8.28 (fig. 19).

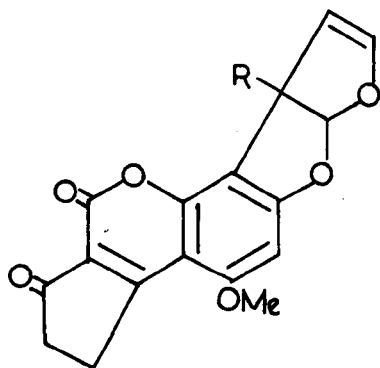
The extinction coefficient of methyl tiglate was determined, λ_{max} . 217 nm. ϵ 8,890, using this value the molecular weight of azadirachtin was estimated to be 700 from its ultraviolet absorption maximum.

The n.m.r. spectrum of azadirachtin showed two doublets at τ 3.55 and τ 4.95, of one proton each, which appeared to be coupled together with a coupling constant of 3 Hz, this was confirmed by spin decoupling at 100 MHz (fig. 19). The chemical shift of the low field doublet was indicative of a proton on a double bond further deshielded by an adjacent carbonyl group or ether oxygen, the first possibility is ruled out by the absence of ultraviolet absorption other than that of the tiglate ester group. The low value of the coupling constant is consistent with the vinyl ether being part of a ring system, in 2,3 dihydropyran³⁴ $J = 6$ Hz and in 2,3 dihydrofuran³⁵ $J = 2.6$ Hz.

Very few naturally occurring compounds are known which contain the dihydrofuran system. A group of compounds have been isolated which contain the dihydrofurobenzofuran system and which show toxic and carcinogenic potency in many animal species. The first of these structures to be established was that of sterigmatocystin³⁶ (22) from *Aspergillus versicolor*.

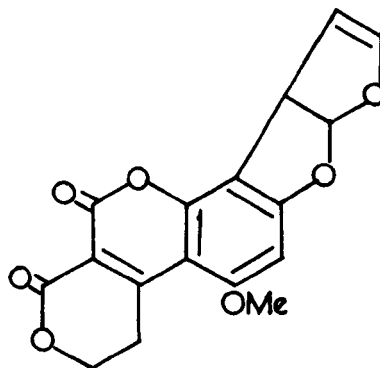


Later the structures of the aflatoxins B₁ (23) G₁ (24)³⁷ and M₁ (25)³⁸ from *Aspergillus flavus* were elucidated.



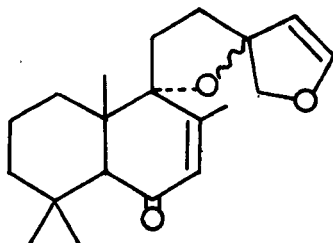
(23) R = H

(25) R = OH



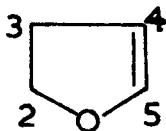
(24)

More recently McCrindle *et al.*³⁹ have isolated from *Solidago canadensis* a mixture of epimeric ethers to which they assigned the structure (26).



(26)

The n.m.r. spectral data for the protons of the dihydrofuran system of these compounds and the parent compound 2,3 dihydrofuran are summarised below.



	H-5	H-4	H-2	$J_{4,5}$	$J_{3,5}$
2,3 dihydrofuran ³⁵	τ 3.78	τ 5.18	τ 5.8	2.6 Hz.	2.6 Hz.
Aflatoxin B ₁ ³⁷	τ 3.48	τ 4.47	τ 3.11	2.5 Hz.	2.5 Hz.
Aflatoxin M ₁ ³⁸	τ 3.17	τ 4.36	τ 3.54	3 Hz.	-
Spiro-ether mixture ³⁹	τ 3.5	τ 4.9	τ 5.8	not quoted.	

The spiro-ether is a mixture of two isomers, which complicates the n.m.r. spectrum, however in this case there are no protons in the 3-position of the dihydrofuran and the only coupling of H-4 and H-5 is between each other, so that the absorptions due to H-4 and H-5 each consist of two overlapping doublets. As is the case with all these examples no coupling is observed between the C-2 proton or protons and H-4 and H-5, but in each of the cases where there is a proton in the 3-position H-4 and H-5 are further coupled.

In the n.m.r. spectrum of azadirachtin the doublets occur at τ 3.55 and τ 4.95, which are closer to the values observed for the olefinic protons of the dihydrofuran ring in 2,3 dihydrofuran and the spiro-ether mixture from *Solidago canadensis*, the corresponding absorptions in the

dihydrofurobenzofuran occur at lower field because of the benzene ring. It would appear that if a dihydrofuran system is present in azadirachtin it must be fully substituted in the 3-position because the absorptions assigned to the olefinic protons appear as simple doublets.

Now that the presence of at least two double bonds had been established in azadirachtin an attempt was made to hydrogenate the molecule. Treatment of azadirachtin in glacial acetic acid at room temperature with hydrogen at atmospheric pressure in the presence of Adam's platinum catalyst did not have any effect, however in ethyl acetate with hydrogen at a pressure of 50 lbs/sq.in. using the same catalyst, hydrogenation occurred to give a product which had a lower R_f than azadirachtin on thin layer chromatography and could be purified by preparative layer chromatography.

The mass spectrum of azadirachtin was first recorded on an A.E.I. M.S.9 instrument by Dr. V.P. Williams at the Shell Laboratory for Chemical Enzymology, Sittingbourne; the highest ion of any significance was at 642 and this was taken to be the molecular ion, an accurate mass determination of this ion gave the molecular formula $C_{29}H_{38}O_{16}$ which was reported in a preliminary communication.⁴⁰ This was later found to be incorrect, when this ion and a number of other significant ions were subjected to accurate mass determination (see Experimental Section), the ion m/e 642 was found to correspond to a molecular formula of $C_{33}H_{38}O_{13}$. Ions at m/e 627 and 611 corresponded to the loss of $.CH_3$ and $.OCH_3$ from the methyl ester groups.

When the mass spectrum of a different sample of azadirachtin was recorded on the Hitachi-Perkin Elmer RMU.6 instrument, (fig. 3) ions at 702, 688, 670 and 660 were observed corresponding to extremely weak ions in the original spectrum which were thought to be impurity, in this case the ion m/e 670 was in greater abundance than the ion m/e 642.

Further evidence for the molecular weight of azadirachtin being greater than 642 came from the mass spectrum of the hydrogenation product (fig. 4) which showed significant ions at 690, 672, 662 and 644 all two mass units higher than significant ions in the mass spectrum of azadirachtin, indicating that the hydrogenation product is the dihydro-derivative of azadirachtin.

The doublets in the n.m.r. spectrum of azadirachtin, which have been assigned to the olefinic protons of a dihydrofuran ring, were no longer present in the n.m.r. spectrum of dihydro-azadirachtin, (fig. 5) therefore it is this double bond which has been hydrogenated, there was an increase in the absorption in the region of τ 5.9 and τ 7.8 due to methylene groups formed in this hydrogenation. The absorptions in the n.m.r. spectrum due to the tiglate ester group remained, showing that the double bond of the tiglate ester group had not been hydrogenated, further confirmation of this was given by the ultraviolet spectrum which showed λ_{max} . 219 nm. and also the infrared spectrum showed the absorption at 1655 cm^{-1} assigned to the double bond of the tiglate ester group, however the absorption at 1625 cm^{-1} in the spectrum of azadirachtin was not present in the spectrum of dihydro-azadirachtin, indicating that this absorption

Mass Spectrum of Azadirachtin

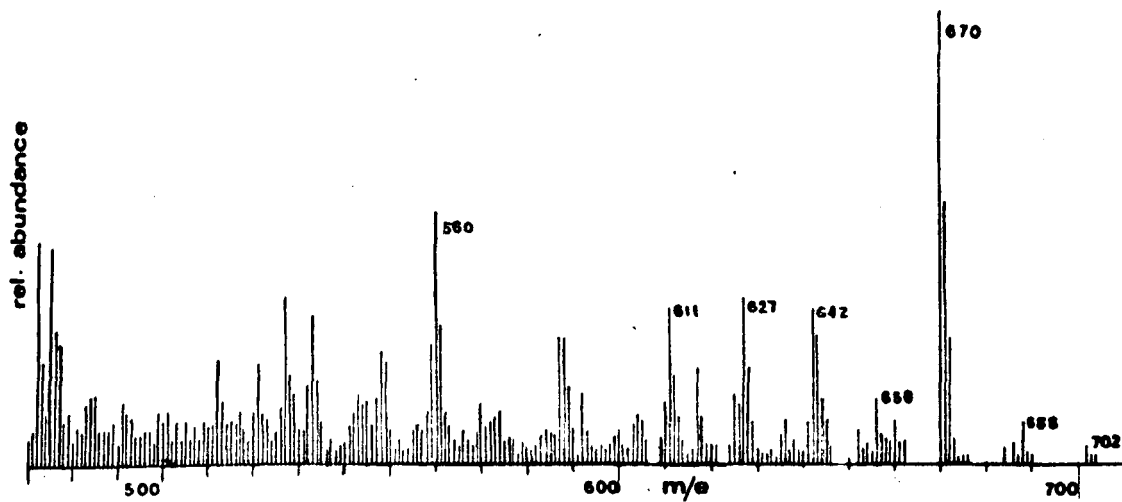


Figure 3

Mass Spectrum of Dihydro-azadirachtin

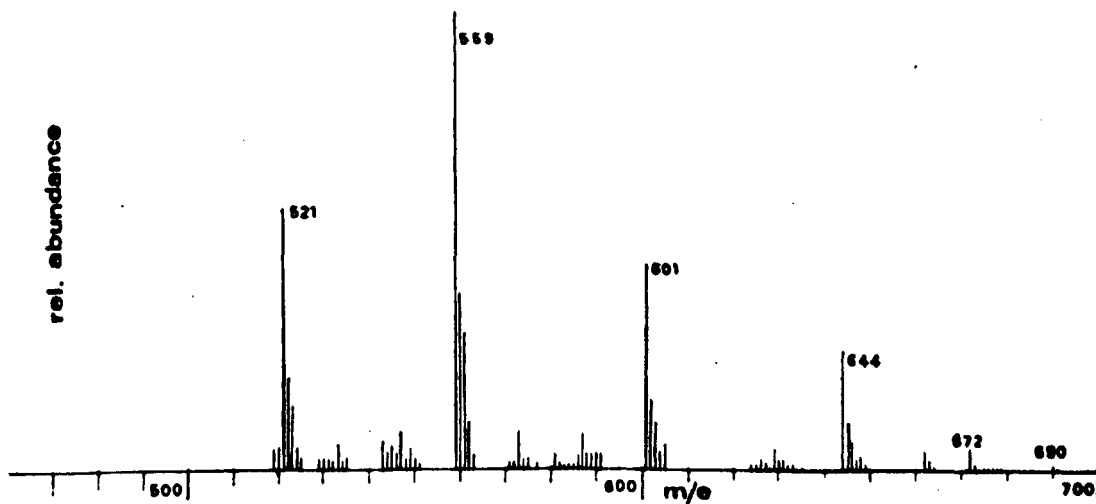


Figure 4

60MHz. N.M.R. Spectrum of Dihydro-azadirachtin

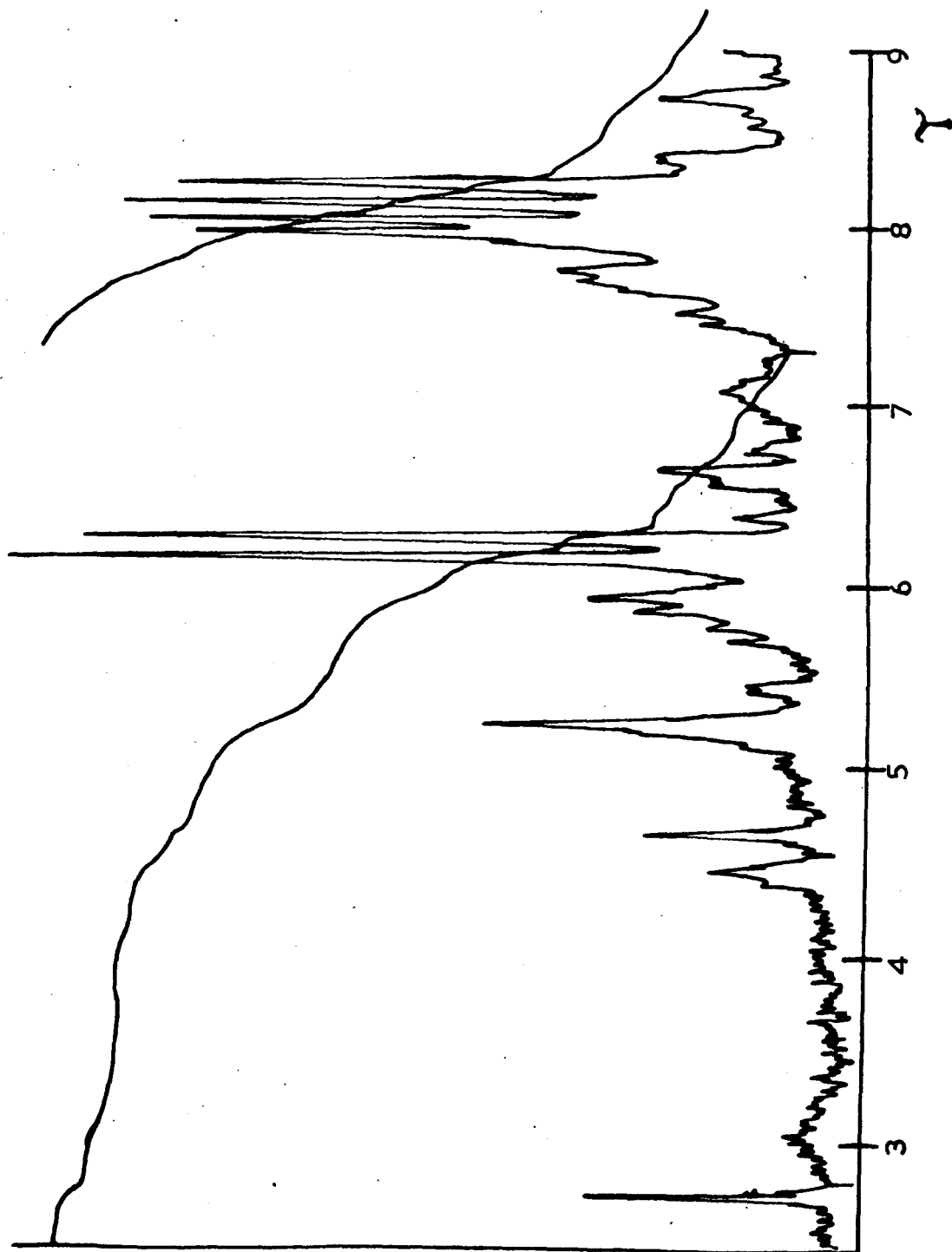
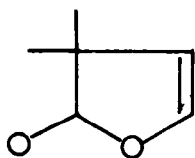


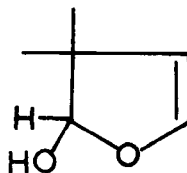
Figure 5

is due to the double bond which has been assigned to a dihydrofuran ring.

The one proton singlet at $\tau 4.4$ in the n.m.r. spectrum of azadirachtin was shifted to $\tau 4.7$ in the spectrum of dihydro-azadirachtin, so it appears that this absorption is due to a proton near to the double bond. If the dihydrofuran ring were oxygenated in the C-2 position either as a hemi-acetal (27) or acetal system (28), it has already been suggested that the 3-position of the dihydrofuran ring is fully substituted, so that the proton next to the oxygen would appear as a sharp singlet; this proton being deshielded by two adjacent oxygen atoms and also to a small extent by the double bond. The chemical shift of this proton is not comparable with the chemical shifts of the proton in the same position in the aflatoxins because these are affected by the benzene ring.

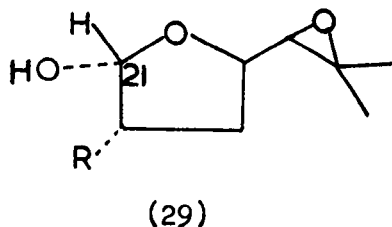


(28)



(27)

If the system is a hemi-acetal the hydrogenation product would contain a hemi-acetal system comparable with the hemi-acetal system (29) in melianone^{26,27} (11).



The H-21 proton in melianone absorbs at τ 4.62 which is very similar to the position of the sharp singlet in the spectrum of dihydro-azadirachtin. However it is unlikely that there would be any great difference in the chemical shift of the H-2 proton in the hemiacetal or acetal systems, so that neither possibility can be ruled out on this evidence.

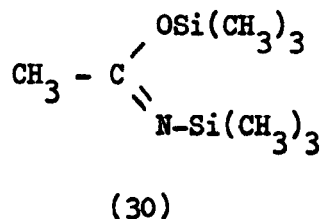
The mass spectrum of hydrogenated azadirachtin gave further indication that the molecular weight of azadirachtin was higher than 642, but it was still impossible to arrive at the true value. It was thought that the difficulty encountered in obtaining a molecular ion may have been due to the involatility of azadirachtin and dihydro-azadirachtin, so it was considered necessary to try to prepare a more volatile derivative of azadirachtin which would perhaps give a molecular ion on mass spectrometry.

Early attempts on a small scale to prepare an acetate of azadirachtin which would probably be more volatile did not appear to meet with success, several methods were tried including the action of acetic anhydride, acetic anhydride-pyridine and acetic anhydride-boron trifluoride. The products obtained were mixtures, the major components

of which had R_F values less than that of azadirachtin on thin layer chromatography, it was expected that an acetate of azadirachtin would be less polar than azadirachtin and have a greater R_F . Because of this and the limited amount of azadirachtin available the reactions were not repeated on a larger scale at this stage.

Trimethyl-silyl ether derivatives of alcohols⁴¹ have been used extensively for purification and identification purposes in recent years. The increased volatility of the trimethyl-silyl ether over that of the parent alcohol very often lends itself to gas chromatographic separation and mass spectrometry in which the trimethyl-silyl group in many cases appears to direct characteristic fragmentation patterns.

Bis(trimethyl-silyl) acetamide, (30)⁴² a reagent obtainable commercially which readily forms silyl derivatives under mild conditions, was used in an attempt to form the trimethyl-silyl derivative of azadirachtin.



Excess of this reagent was added to a chloroform solution of azadirachtin, after a few minutes the volatile components were pumped off and the gummy residue was subjected to mass spectrometry. The mass spectrum showed ions at considerably higher m/e to those observed in the

spectrum of azadirachtin and the highest peaks appeared at m/e 864. Dihydro-azadirachtin treated similarly gave a mass spectrum with the highest peak at m/e 866.

It was thought that the trimethyl-silyl ether derivative would be moisture sensitive and therefore it would not be practicable to purify the product by preparative layer chromatography. However the reaction was repeated on a larger scale and an attempt was made to purify the product by preparative layer chromatography; by extracting the silica with chloroform instead of methanol to reduce the risk of hydrolysis, the pure trimethyl-silyl ether derivative of azadirachtin was isolated.

The n.m.r. spectrum of this derivative (fig. 6) showed two absorptions at τ 9.8 and τ 9.9, the integrated spectrum clearly showed that two trimethyl-silyl ether groups had been introduced and that, therefore, since the general appearance of the n.m.r. spectrum was unchanged, there are two hydroxyl groups in azadirachtin. The infrared spectrum did not show the broad absorption attributed to hydroxyl, present in the spectrum of azadirachtin.

The mass spectrum of the trimethyl-silyl ether derivative is shown in fig. 7, the fragmentation can be partially explained. Loss of a methyl radical would give the ion m/e 849, such α -cleavage commonly occurs in trimethyl-silyl ethers.⁴³

60MHz. N.M.R. Spectrum of Bis(trimethyl-silyl)azadirachtin

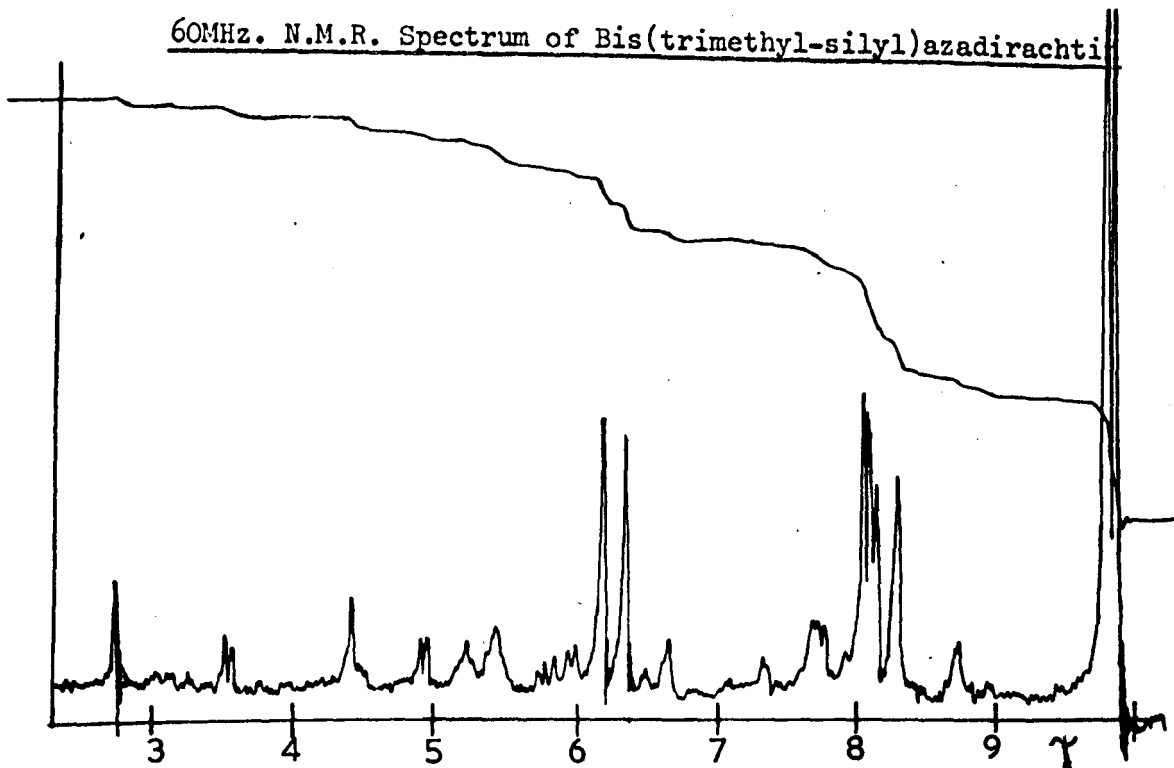


Figure 6

Mass Spectrum of Bis(trimethyl-silyl)azadirachtin

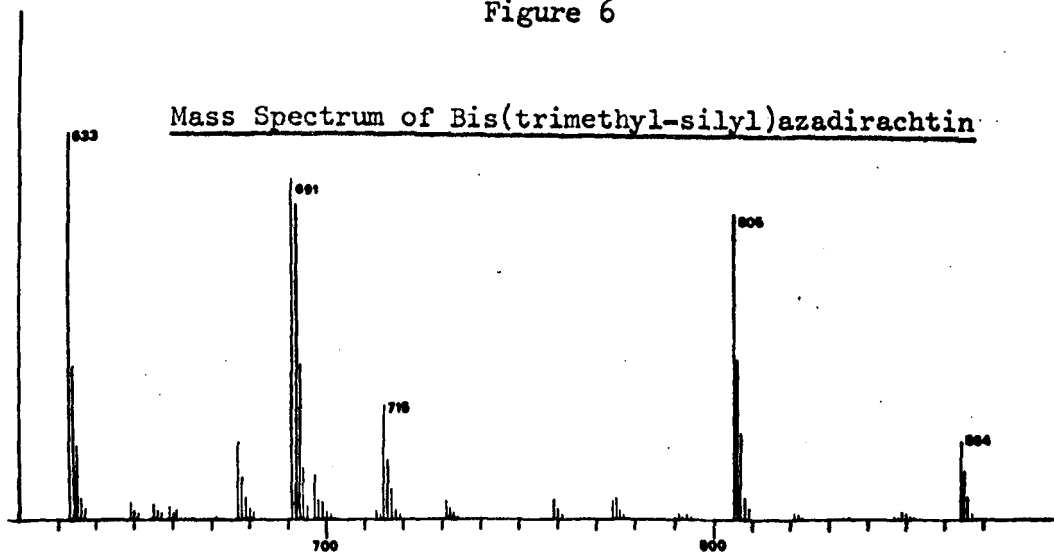
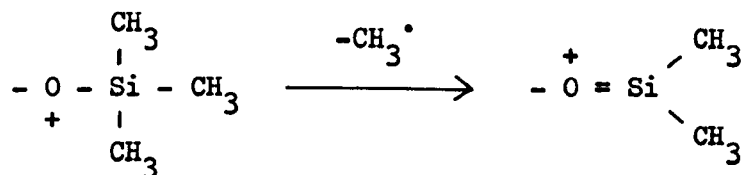


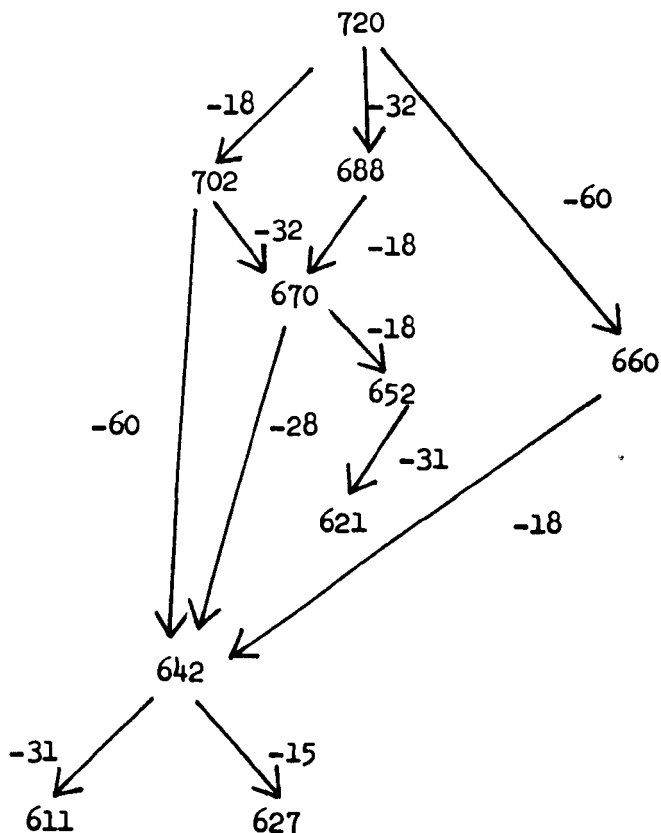
Figure 7



Loss of acetyl would give the ion at m/e 821, alternatively loss of an acetoxyl radical would give the ion m/e 805. The loss of 90 mass units from m/e 805 to m/e 715 could be explained by loss of trimethyl-silanol.⁴³

A molecular weight of 864 for a bis(trimethyl-silyl) ether derivative corresponds to a molecular weight of 720 for azadirachtin.

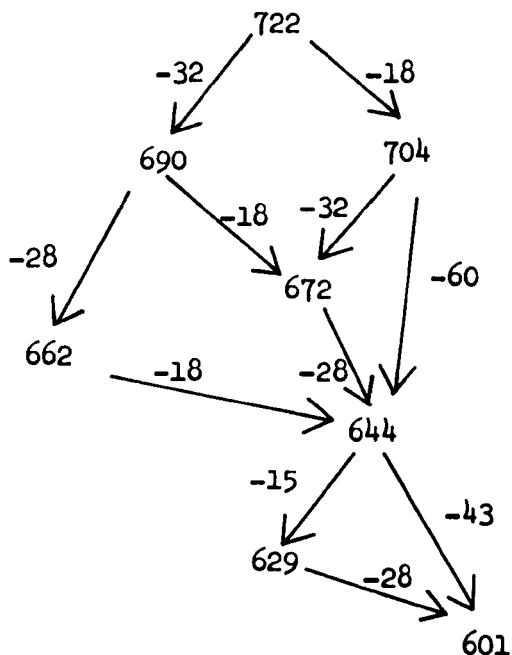
Accurate mass determinations of the m/e 864 ion were carried out by Dr. V.P. Williams and the Physico-Chemical Measurements Unit, Harwell. Both determinations gave a value for the molecular weight corresponding to a formula $\text{C}_{41}\text{H}_{60}\text{O}_{16}\text{Si}_2$, this was in agreement with the analytical data and gives a molecular formula for azadirachtin of $\text{C}_{35}\text{H}_{44}\text{O}_{16}$. The chart below shows how the ions above m/e 600 in the mass spectrum of azadirachtin can be correlated with a molecular ion of 720.



Hence all of the high mass peaks in the mass spectrum can be accounted for by loss of fragments already recognized in the structure of azadirachtin, loss of water, 18, acetic acid, 60, methanol, 32 and methoxyl radical, 31.

Assuming the loss of one molecule of water and one molecule of acetic acid from $C_{35}H_{44}O_{16}$ gives a formula of $C_{33}H_{38}O_{13}$ for the 642 ion which agrees with the formula determined earlier.

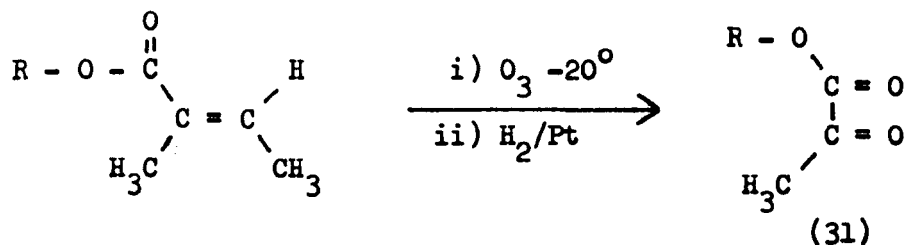
In a similar way the high m/e ions in the mass spectrum of dihydro-azadirachtin can be accounted for by the loss of fragments already identified in the molecule. The loss of 43 mass units being due to loss of acetyl radical.



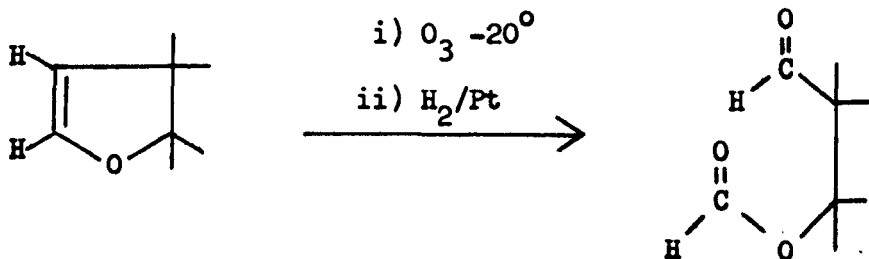
The analytical results for azadirachtin were not very satisfactory. The carbon and hydrogen values of a sample which had been recrystallized from carbon tetrachloride were lower than required for $C_{35}H_{44}O_{16}$. When the same sample was subjected to mass spectrometry it was evident that some carbon tetrachloride had remained trapped in the sample even after heating at 100° under vacuum. The presence of this solvent would tend to lower the analysis figures. The results obtained for a sample of azadirachtin purified by preparative layer chromatography were high, being just outside the acceptable experimental limits. However all the samples of azadirachtin purified by this method showed an absorption in their n.m.r. spectra which was thought to be due to hydrocarbon grease, the presence of such an impurity would tend to give high carbon and hydrogen analyses.

The presence of at least two double bonds in azadirachtin having been established, methods of oxidation of these were attempted. Treatment of azadirachtin in refluxing acetone with potassium permanganate, followed by esterification of the product with diazomethane, gave a mixture which on thin layer chromatography did not show any well defined components.

Treatment of azadirachtin in ethyl acetate at -20° with ozonized air followed by reduction with hydrogen in the presence of Adam's platinum catalyst gave a crude product the n.m.r. of which is shown in fig. 8. This showed an absorption at $\tau 7.5$ which could be assigned to the methyl protons of a pyruvate ester group (31) formed from the tiglate ester.



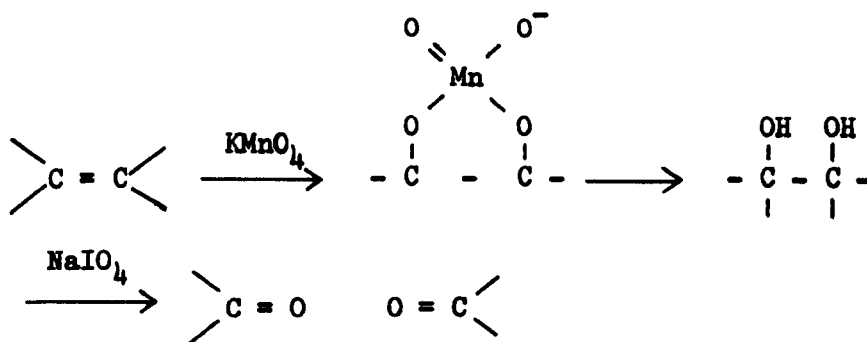
The presence of absorptions at $\tau 2.2$ and $\tau 0.4$, which could be assigned to the protons of a formate ester group and aldehyde group respectively, are further evidence for the presence of a vinyl ether forming part of a ring structure as in the dihydrofuran system. Ozonolysis of such a system would produce a formate ester and aldehyde group.



An attempt was made to purify the ozonolysis product by preparative layer chromatography, but on eluting the chromatogram no clear bands were visible. It was thought that this may have been the result of the aldehyde group being oxidized to the carboxylic acid whilst on the plate. In view of this result another approach was sought.

A reagent which has been used to bring about oxidation of olefins similar to that achieved by ozonolysis is the Lemieux reagent,^{44, 45, 46, 47} which consists of a dilute aqueous solution of sodium metaperiodate with a catalytic quantity of potassium permanganate. For olefins which are not water soluble the reagent has been used with the olefin dissolved in a water miscible solvent such as dioxan, pyridine or t-butanol.

The olefin is first oxidized by the potassium permanganate to the cis-diol which is then cleaved by the sodium metaperiodate to give aldehydes or ketones, any aldehydic products may then be oxidized by the potassium permanganate to carboxylic acids. Manganese in its lower oxidation state is oxidized by the sodium metaperiodate back to its original oxidation state.



When a solution of azadirachtin in t-butanol was treated with the Lemieux reagent a product was isolated by acidification and extraction into chloroform. The n.m.r. spectrum of this material (fig. 9) was very similar to that of the crude ozonolysis product, except that there was no absorption due to an aldehyde proton, this was to be expected since any aldehyde produced by cleavage of a double bond would be oxidized by the potassium permanganate to a carboxylic acid. The infrared spectrum of this product showed a very broad absorption from $3500-2500\text{ cm}^{-1}$ characteristic of the hydrogen bonded OH group of a carboxylic acid. On thin layer chromatography in ether-acetone (4:1) the product gave an elongated spot at the origin, characteristic of a carboxylic acid.

Because carboxylic acids do not run very well on thin layer chromatography, the product was dissolved in chloroform and treated with excess ethereal diazomethane, the n.m.r. spectrum of the esterified product showed an increase in intensity of the absorption in the methyl ester region, the absorption assigned to a formate ester proton was still present, that assigned to the methyl protons of the pyruvate ester group was not present, but there was a new absorption at $\tau 8.4$.

Arndt *et al.*⁴⁸ have reported that diazomethane adds across the α -keto group of methyl pyruvate to give the epoxide (32).

60MHz. N.M.R. Spectrum of the Product from Ozonolysis of Azadirachtin

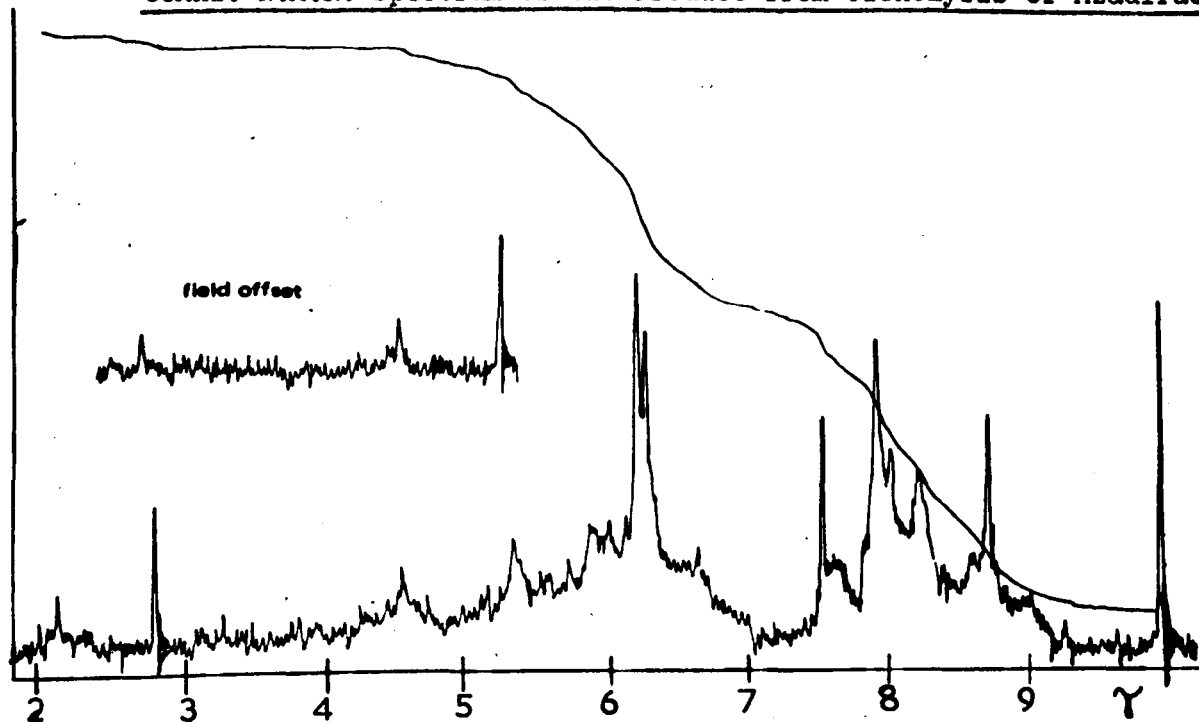


Figure 8

60MHz. N.M.R. Spectrum of the Product from
Lemieux oxidation
of Azadirachtin

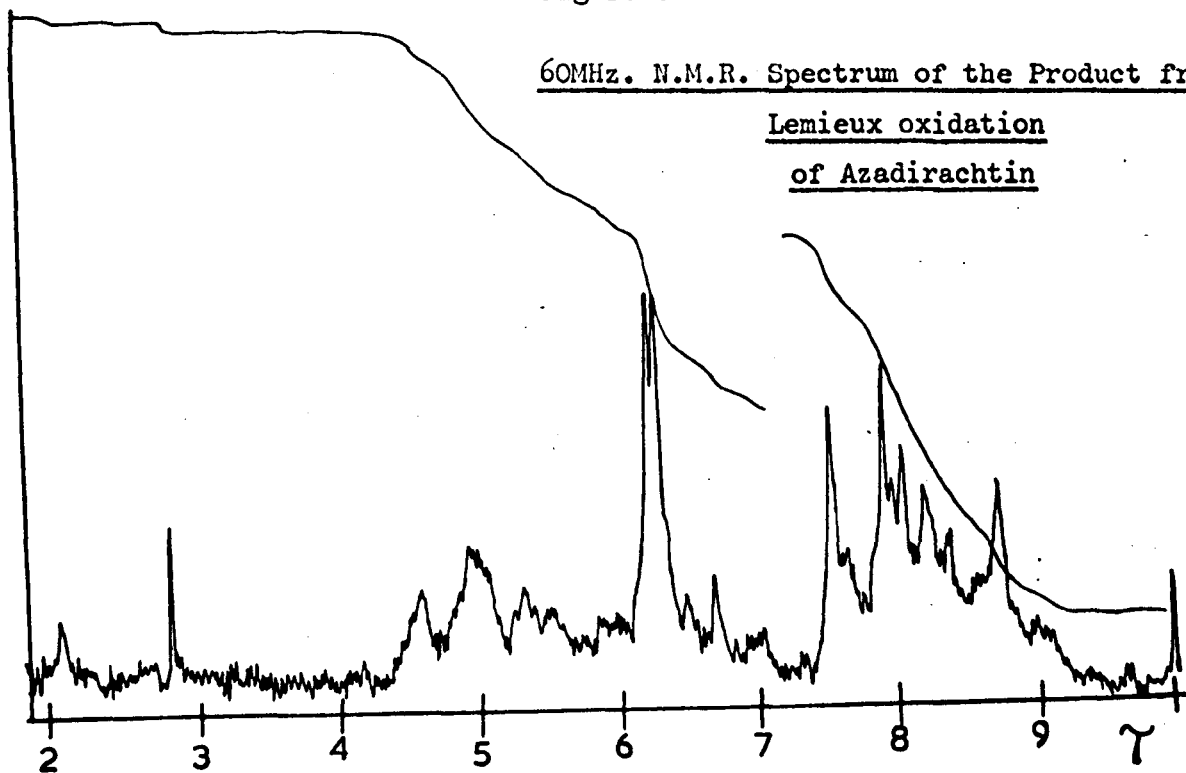
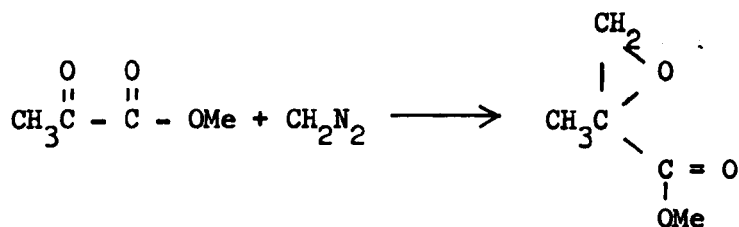


Figure 9



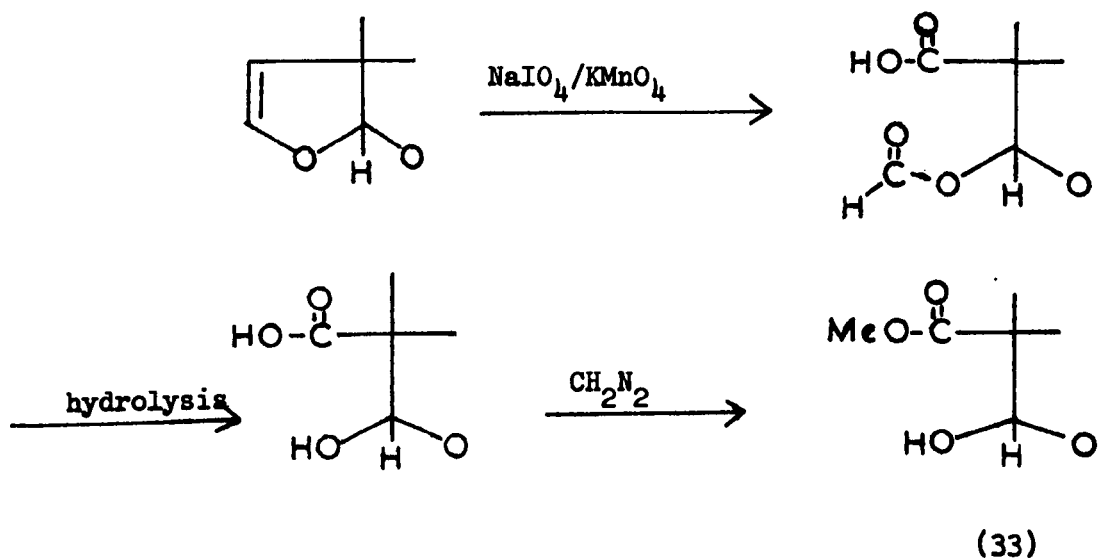
(32)

It seems most likely that this type of reaction had occurred on esterification and so the absorption due to the methyl protons of pyruvyl ester were no longer evident in the n.m.r. spectrum, the corresponding methyl protons of the product being explained by the absorption at $\tau 8.4$.

Pyruvate and formate esters are susceptible to mild hydrolysis, so to avoid the added complication of the formation of an epoxide on esterification, it was decided to try to remove the pyruvate and formate esters selectively before esterification. The crude Lemieux oxidation product was treated with aqueous methanolic potassium bicarbonate at room temperature for 24 hours. The n.m.r. spectrum of the material isolated by acidification, extraction into chloroform and esterification with diazomethane, did not show absorptions at $\tau 2.2$ and $\tau 7.5$ assigned to the protons of formate and pyruvate esters. Thin layer chromatography showed that this product consisted of at least three components, which were separated by preparative layer chromatography.

The spectral evidence for the dihydrofuran ring forming part of a hemi-acetal or acetal system has already been described, further

evidence which tends to rule out the possibility of a hemi-acetal will be described later. Lemieux oxidation of the acetal system followed by hydrolysis and esterification would be expected to give the structure (33).



The products obtained from this reaction all had very similar n.m.r. spectra, but it was not possible to correlate any of these products with the predicted product containing the structural fragment (33).

Azadirachtin has been shown to contain two hydroxyl groups, an attempt was made to oxidize one or both of these groups using Cornforth's reagent⁴⁹ (chromium trioxide-pyridine). After treatment with this reagent at room temperature for two days only starting material was recovered. This suggested that the hydroxyl groups are

both tertiary or highly hindered. This method was chosen rather than the acidic Jones reagent (chromic acid-acetone) because of the sensitivity of azadirachtin to acid.

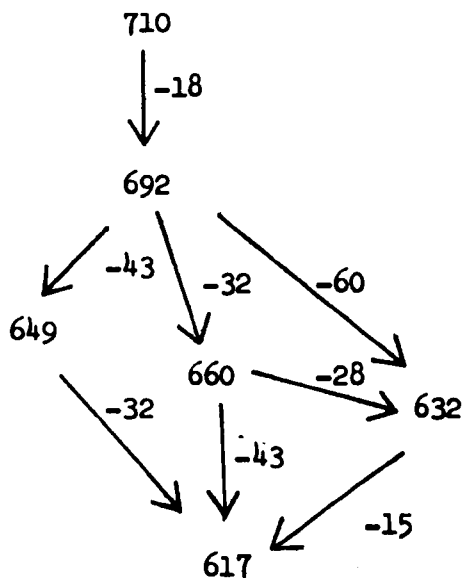
It was thought that if one or more of the ester groups of azadirachtin could be removed by alkaline hydrolysis, the hydroxyl group or groups produced may be susceptible to oxidation with Cornforth's reagent, and so further information on the structure of azadirachtin could be obtained. However early attempts to obtain such a selective hydrolysis product were unsuccessful.

The presence of a double bond in the tiglate ester group of dihydro-azadirachtin made it possible to remove this group selectively. Overton *et al.*⁵⁰ have used osmylation followed by periodate oxidation and mild hydrolysis of the pyruvate ester formed to remove the tiglate ester from swietenine.

Since a pyruvate ester had been obtained on treating azadirachtin with the Lemieux reagent, dihydro-azadirachtin was treated under similar conditions and a single product detigloyl-pyruvyl-dihydro-azadirachtin was formed, which was purified by preparative layer chromatography. The n.m.r. spectrum of this compound (fig. 10) showed a singlet at $\tau 7.5$ which could be attributed to the methyl protons of a pyruvate ester group, the absorptions due to the methyl protons of the acetate groups appeared almost superimposed at $\tau 8.0$, the singlet at $\tau 8.25$, which is obscured by the tiglate ester absorption in the spectrum of azadirachtin and is revealed by spin decoupling (fig. 19), could be

clearly seen.

Dihydro-azadirachtin has a molecular weight of 722 and therefore the pyruvate ester derivative should have a molecular weight of 710. As with azadirachtin and dihydro-azadirachtin a molecular ion was not observed in the mass spectrum, the ion of highest mass observed, m/e 692, corresponded to a loss of water from a molecular ion of 710. All the ions above m/e 600 in the mass spectrum can be accounted for by loss of fragments already known to be present in the molecule, that is loss of water, 18, methanol, 32, acetic acid, 60, and acetyl radical, 43.



Overton *et al.*⁵⁰ found that treatment of the pyruvate ester of swietenine with aqueous sodium bicarbonate solution at 20° for 15 minutes gave detigloylswietenine. When detigloyl-pyruvyl-dihydro-azadirachtin was treated with aqueous methanolic sodium bicarbonate at

room temperature for ten minutes, a product was isolated which was shown to be mainly starting material by thin layer chromatography. Treatment for a further hour gave a product which on thin layer chromatography was found to be mainly a component with a lower R_f than detigloyl-pyruvyl-dihydro-azadirachtin. The $\tau 7.5$ absorption in the n.m.r. spectrum of the crude product due to the methyl protons of the pyruvate ester was considerably less intense than in the spectrum of the starting material, indicating that almost complete hydrolysis of the pyruvate ester group had occurred. Detigloyl-dihydro-azadirachtin was isolated from the mixture by preparative layer chromatography.

The n.m.r. spectrum of detigloyl-dihydro-azadirachtin (fig. 11) showed the absorptions of the methyl ester protons very close together at $\tau 6.20$ and $\tau 6.23$, the absorptions due to the methyl protons of the two acetate groups were superimposed at $\tau 7.95$.

There was no absorption maximum in the ultraviolet spectrum of this derivative, confirming that the absorption in the spectrum of dihydro-azadirachtin is due only to the tiglate ester group. The infrared spectrum showed a much sharper absorption in the carbonyl region at 1740 cm^{-1} , than in the spectrum of azadirachtin, because the absorption at 1710 cm^{-1} due to the tiglate ester group was not present.

The expected molecular weight of detigloyl-dihydro-azadirachtin is 640. The ion of highest mass observed in the spectrum of this compound was at m/e 622, which corresponds to loss of water from the molecular ion. All the higher mass ions in this spectrum can be accounted for by

60MHz. N.M.R. Spectrum of Detigloyl-pyruvyl-dihydro-azadirachtin

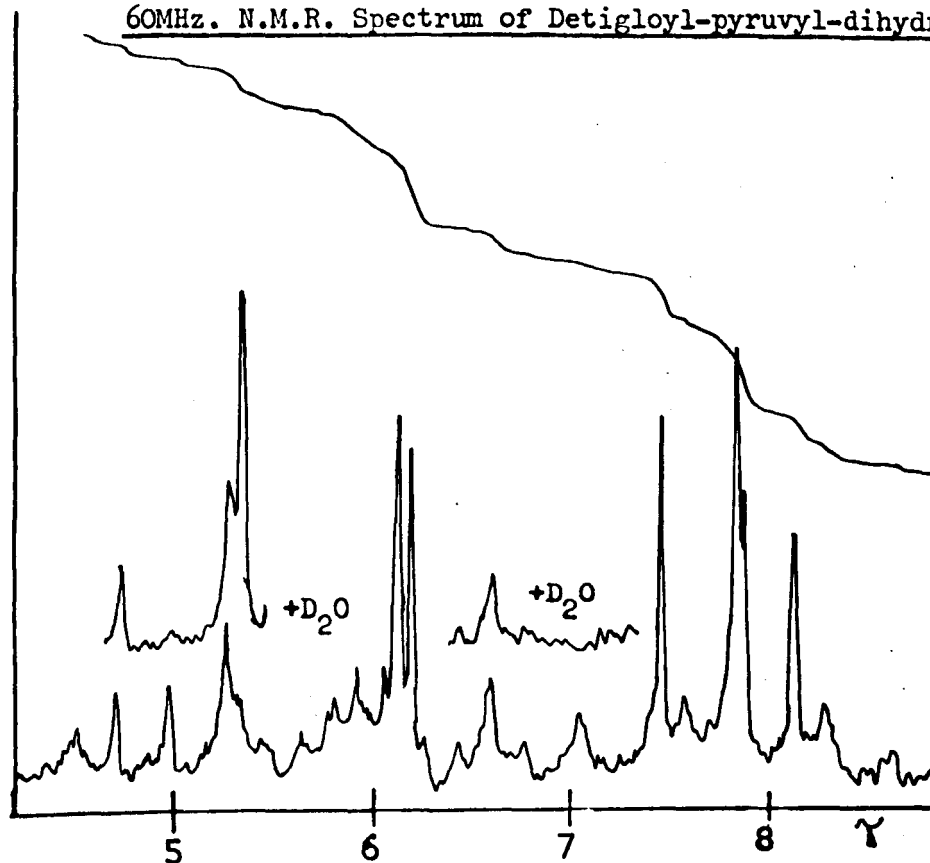


Figure 10

60MHz. N.M.R. Spectrum of Detigloyl-dihydro-azadirachtin

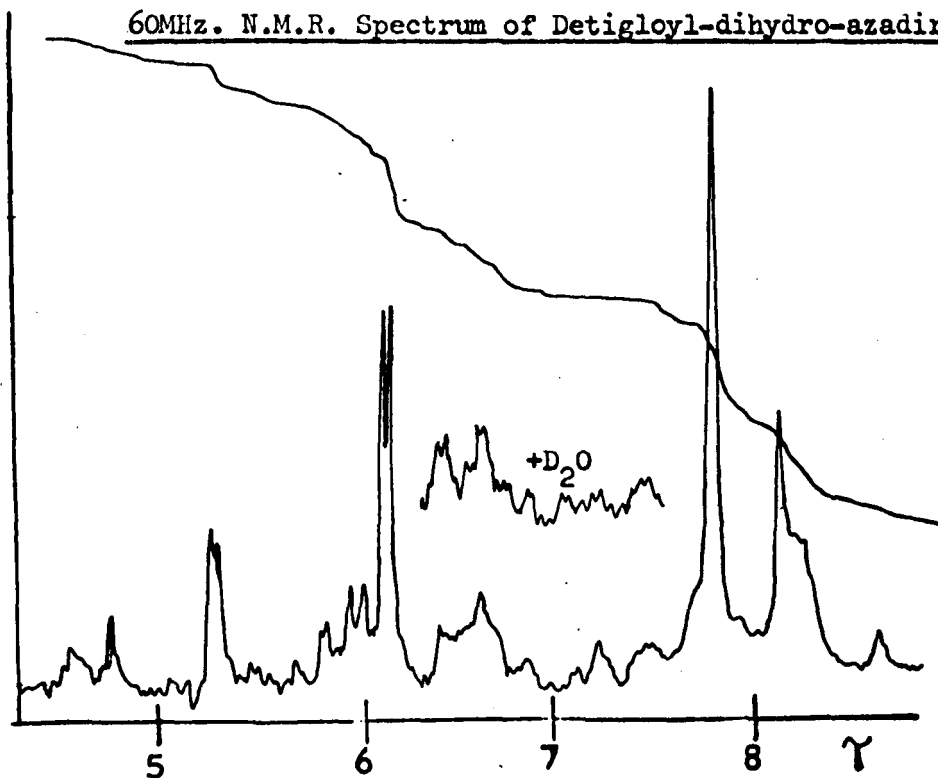
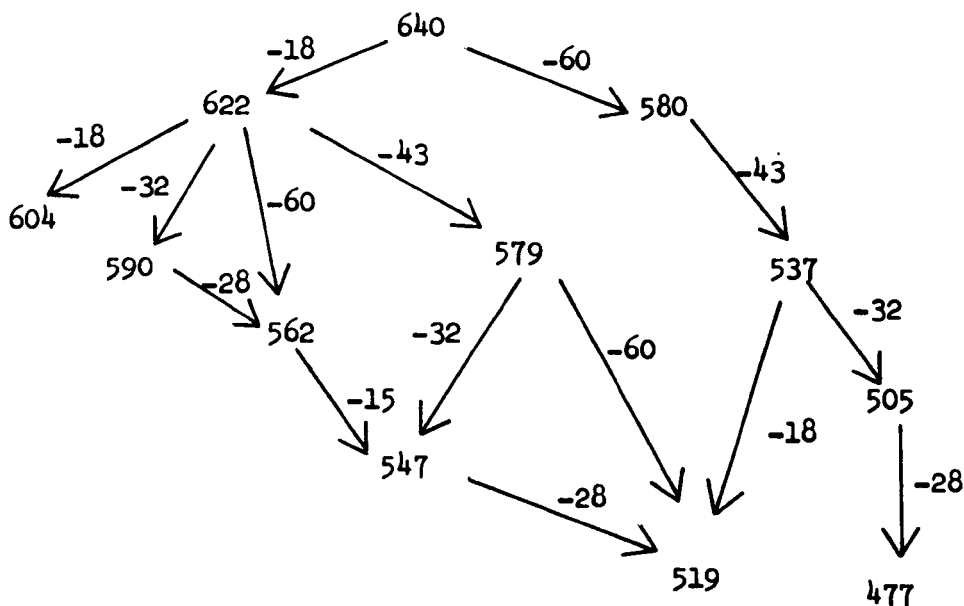


Figure 11

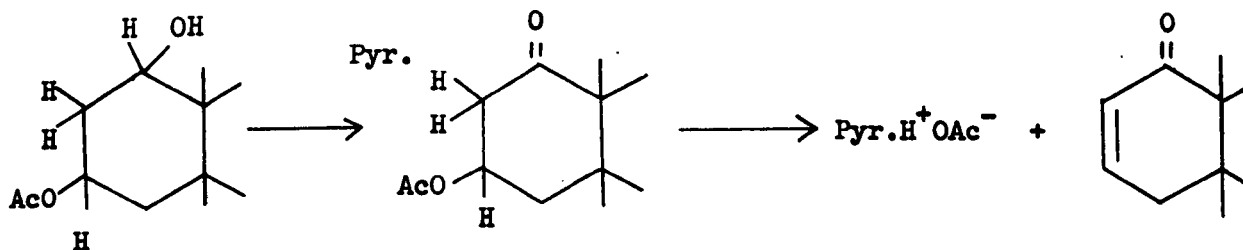
the loss of fragments, which have already been identified in the molecule.



Detigloyl-dihydro-azadirachtin contains an additional hydroxyl group, so an attempt was made to oxidize this compound using Cornforth's reagent.⁴⁹ Treatment for 65 hours at room temperature gave a product which on thin layer chromatography in ether-acetone (4:1) appeared to be a mixture of starting material and one other less polar component, these were separated by preparative layer chromatography. The less polar component, however, was found to be a mixture of two components on thin layer chromatography in ethyl acetate, so a further preparative layer chromatography was carried out, eluting with ethyl acetate. The major component was isolated as a slightly yellow glass which was identified as

an $\alpha\beta$ -unsaturated ketone, by absorptions in its accumulated n.m.r. spectrum (fig. 12) at $\tau 2.9$ and $\tau 3.8$ characteristic, of the β and α protons respectively of such a ketone. An absorption in the infrared spectrum at 1690 cm^{-1} was characteristic of a six-membered ring $\alpha\beta$ -unsaturated ketone as was the absorption in the ultraviolet spectrum, $\lambda_{\text{max.}} 225\text{ nm. } E_{1\%}^{1\text{cm.}} 2,300$.

It is possible that the $\alpha\beta$ -unsaturated ketone could have been formed by the oxidation of the new hydroxyl group followed by loss of acetate, in the mildly basic medium, in a system where an acetate group is in the β -position to the hydroxyl. This would mean that the tiglate ester group in azadirachtin is β to an acetate group, this conclusion is supported by spin-decoupling evidence.



Schutt and Tamm⁵¹ have reported that a similar reaction occurs when the acetate (34) is subjected to the mildly basic conditions of an alumina column.

60MHz. N.M.R. Spectrum of the Product from Oxidation of Detigloyl-dihydro-azadirachtin
with Cornforth's Reagent

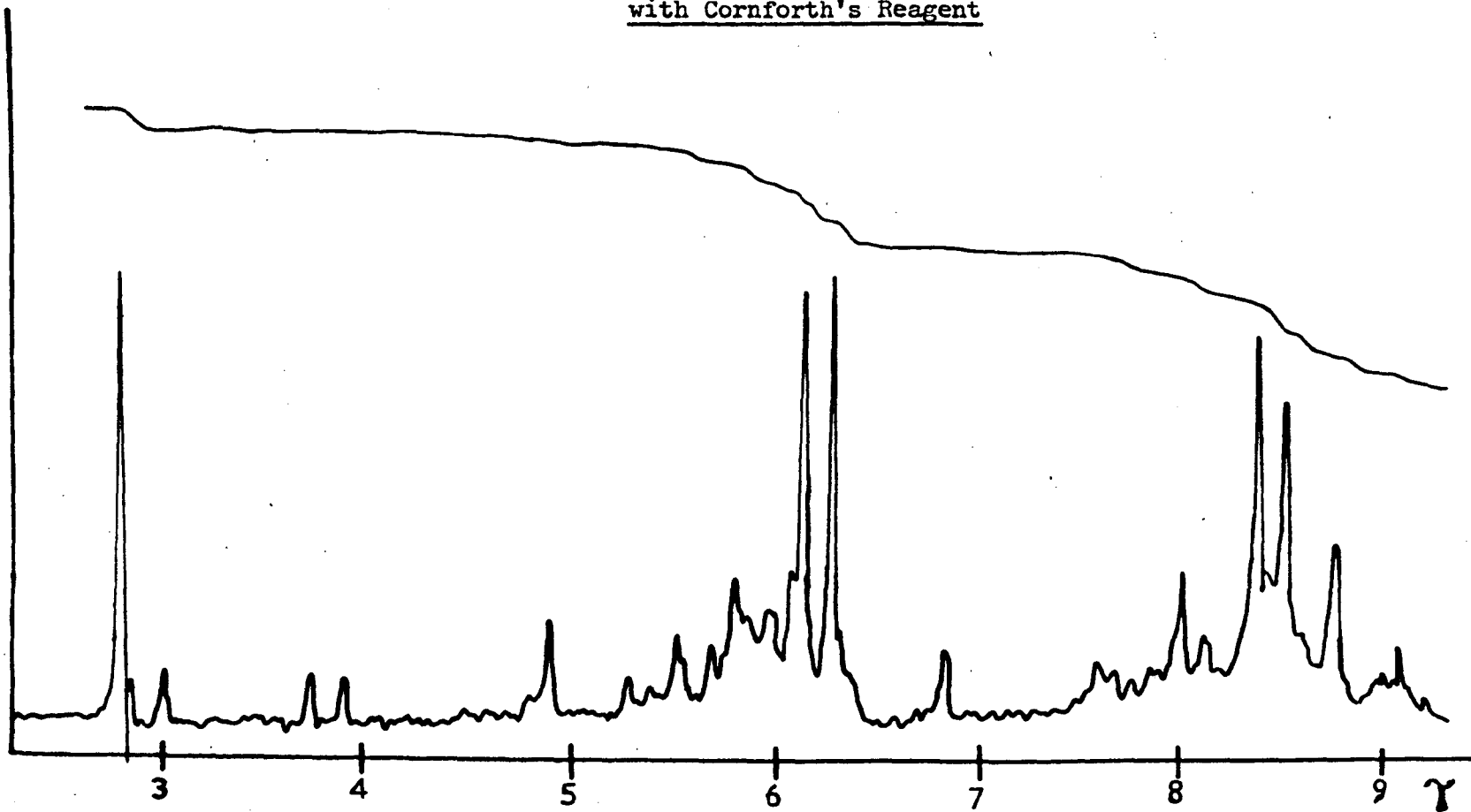
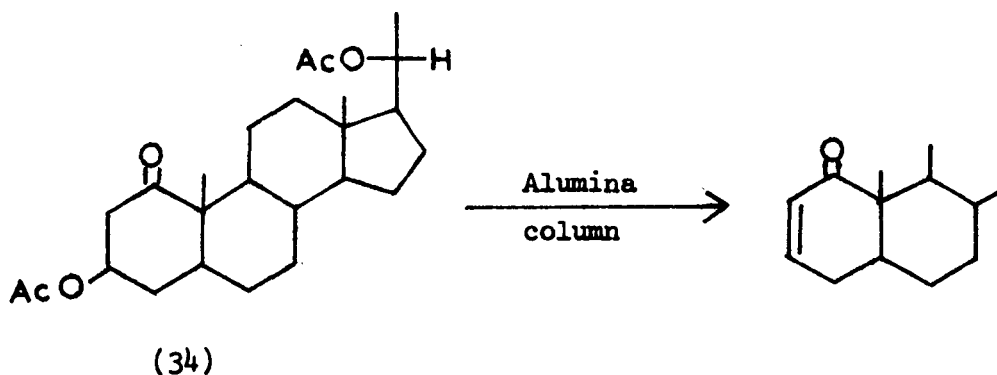
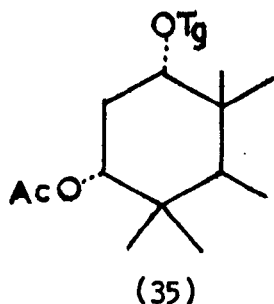


Figure 12



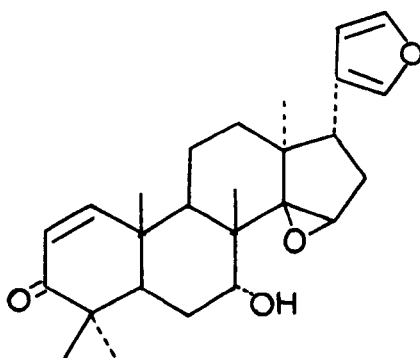
The absorptions in the n.m.r. spectrum of the $\alpha\beta$ -unsaturated ketone at τ 8.3 and τ 8.5 were at rather too high field to be due to acetate so it appeared that both acetate groups had been lost in this reaction. The absorption at τ 8.35 corresponded to the singlet in the n.m.r. spectrum of the starting material, but the absorption at τ 8.5 was unaccounted for.

The structural feature (35) is present in salannin^{19,20} (2)



The corresponding $\alpha\beta$ -unsaturated ketone to salannin would be a $\Delta^{2,3}$ -1-ketone such as is found in nimbin^{16,17} (1). The chemical shifts of the H-2 and H-3 protons in nimbin are τ 4.16 and τ 3.68, however

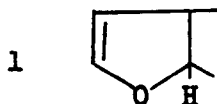
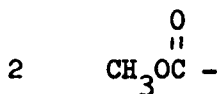
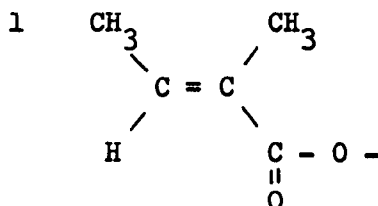
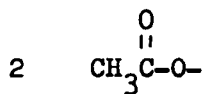
the corresponding shifts in the $\alpha\beta$ -unsaturated ketone derived from detigloyl-dihydro-azadirachtin are τ 3.8 and τ 2.85. These correspond more closely to the chemical shifts of the H-2 and H-1 protons in a $\Delta^{1,2}$ -3-ketone such as the compound⁵² (36) which are at τ 2.82 and τ 4.18.



(36)

The mass spectrum of the $\alpha\beta$ -unsaturated ketone was not very informative, because ions were observed of higher mass than the molecular ion of the starting material, apparently some impurity was present. No further work has been carried out on this reaction product to completely relate the structure of this $\alpha\beta$ -unsaturated ketone to that of azadirachtin.

The groups already assigned to the structure of azadirachtin are summarised below.



These assignments leave three oxygen atoms of the molecular formula $\text{C}_{35}\text{H}_{44}\text{O}_{16}$ to be accounted for.

Treatment of azadirachtin with sodium borohydride for a short time at room temperature did not produce any reaction, treatment for a longer time gave a complex mixture of products, probably the result of alkaline hydrolysis. The resistance of azadirachtin to sodium borohydride reduction is an indication that either a ketone group is not present or is present in a hindered position.

The failure to form a 2,4-dinitrophenylhydrazone of azadirachtin was to be expected since it is very sensitive to acid conditions; in an

attempt to form a semicarbazone derivative, which requires less acid conditions, only starting material was recovered, this was further indication of the absence of a ketone group.

To determine whether azadirachtin contained a lactone group, it was treated in ethanol solution with dilute alkali, back titration showed that half an equivalent of base had been consumed, assuming the molecular weight of azadirachtin to be 720. Acidification and extraction with chloroform gave a product consisting of at least two components, from which the major component was isolated by preparative layer chromatography. This component had an R_f on thin layer chromatography very similar to that of azadirachtin. Its n.m.r. spectrum closely resembled that of azadirachtin except that the high field methoxyl absorption was very much reduced in intensity, and there was a larger absorption in the $\tau 8.7$ region. This suggested that the component was the compound in which one of the methyl ester groups had been replaced by an ethyl ester group. This could have been formed by hydrolysis of the methyl ester group having taken place in alkaline solution followed by esterification with ethanol on acidification. The less intense high field methoxyl absorption in the n.m.r. spectrum could have been caused by the presence of some azadirachtin as impurity. The mass spectrum showed ions corresponding to azadirachtin and also additional ions, one at 716 which would correspond to the loss of 18 mass units from a molecular ion m/e 734, the expected molecular weight of the ethyl ester.

Since only half an equivalent of base was consumed in this

reaction there is no evidence for the presence of a lactone.

It has already been mentioned that early attempts at alkaline hydrolysis of azadirachtin were unsuccessful. Azadirachtin dissolved in methanol was treated with 5% potassium hydroxide solution for two hours, acidification and extraction into ethyl acetate gave a product, sparingly soluble in chloroform, which behaved on thin layer chromatography like a carboxylic acid, giving an elongated spot at the origin on eluting with ether-acetone (4:1).

The product was esterified with diazomethane and the residue obtained was found to be a mixture of five components by thin layer chromatography. These five components were separated by preparative layer chromatography. The accumulated n.m.r. spectra of these components were not very informative, they consisted of rather ill-defined peaks. None of the products appeared to be the expected product from the removal of one or more of the ester groups.

The alkaline hydrolysis was later repeated using 2½% potassium hydroxide solution and allowing it to react for one hour. The product was isolated in the same way as before except that the acidified solution was continuously extracted with ethyl acetate to improve the recovery of material. This time the product after esterification was found to consist of mainly two components by thin layer chromatography. These components were separated by preparative layer chromatography.

The n.m.r. spectrum (fig. 13) of the major component clearly showed it to be azadirachtin from which one of the acetate groups had been removed. The shift of the one proton absorption at $\tau 4.5$ in the

60MHz. N.M.R. Spectrum of Deacetyl-azadirachtin

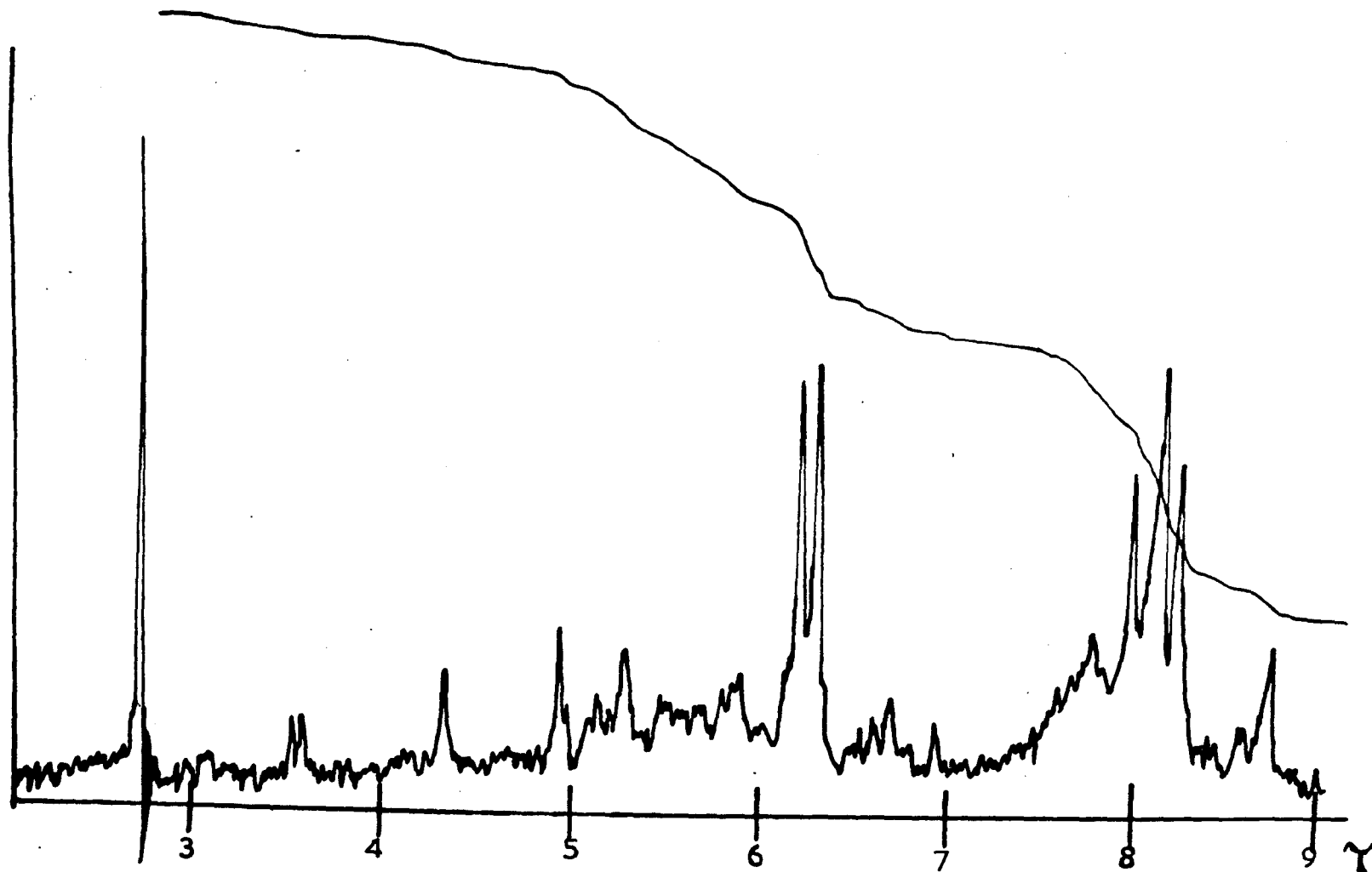
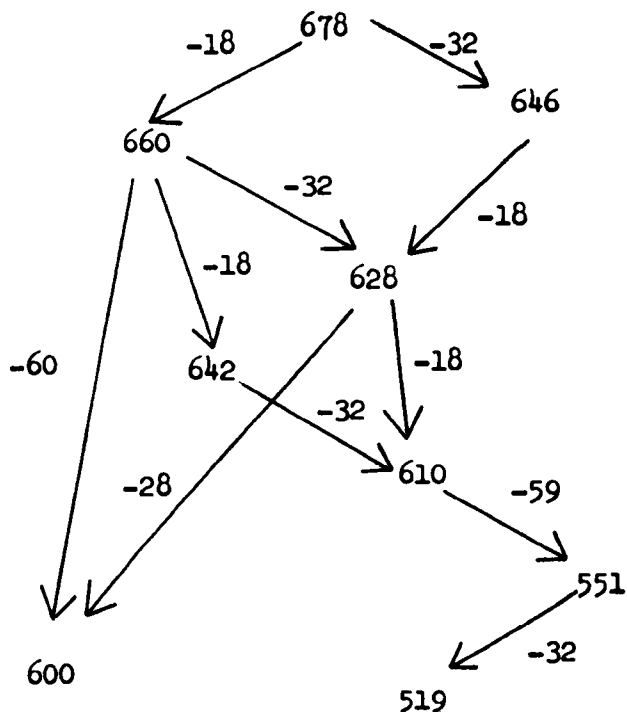


Figure 13

spectrum of azadirachtin to higher field in the spectrum of the deacetyl compound indicates that this absorption is due to a proton on the carbon atom to which the acetate, removed by hydrolysis, is attached in azadirachtin.

The expected molecular weight of this derivative is 678, the ion of highest m/e observed is at 660, this and all the other ions above m/e 500 can be accounted for by the loss of fragments, already assigned to the structure, from the molecular ion m/e 678.

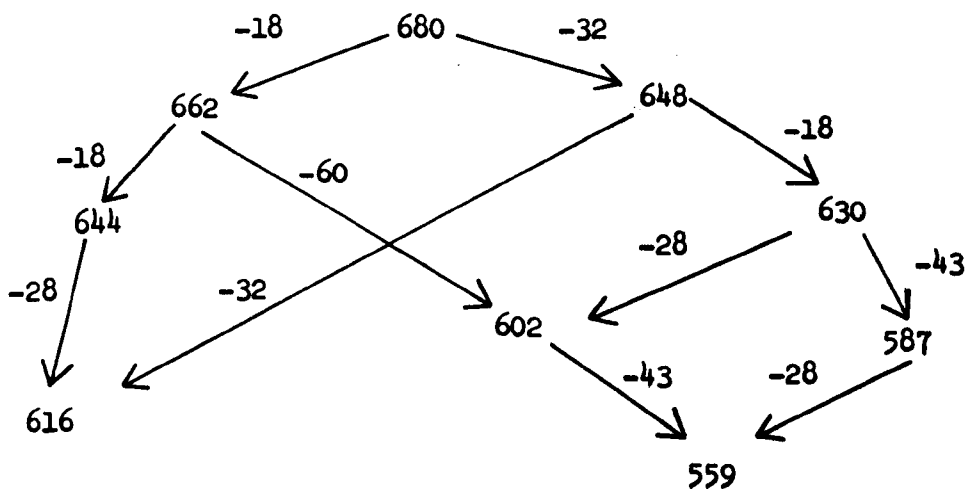


The n.m.r. spectrum of the minor component was complex, the absorptions due to the tiglate and acetate groups were still present, but the doublets of the protons assigned to a dihydrofuran ring were no longer present, and there was an intense absorption at τ 8.7. It would

appear therefore that this was not a straightforward hydrolysis product.

In view of the success in isolating a recognisable hydrolysis product from the alkaline hydrolysis of azadirachtin, alkaline hydrolysis of dihydro-azadirachtin was attempted under the same conditions. Two products were isolated, one was shown by its n.m.r. spectrum (fig. 14) to be dihydro-azadirachtin from which one of the acetates had been removed, corresponding to the product obtained from the alkaline hydrolysis of azadirachtin. Again the proton at $\tau 4.5$ in the spectra of azadirachtin and dihydro-azadirachtin is shifted to higher field in the spectrum of deacetyl-dihydro-azadirachtin, this is further indication that this absorption is due to a proton on the carbon atom to which the acetate, removed by hydrolysis, is attached in azadirachtin.

The expected molecular weight of deacetyl-dihydro-azadirachtin is 680, the molecular ion is not observed in the mass spectrum, the ion of highest mass being at m/e 662. All the ions above m/e 500 in the mass spectrum can be accounted for by the loss of fragments already identified in the molecule.



The other product from the alkaline hydrolysis of dihydro-azadirachtin, which was less polar than either dihydro-azadirachtin or deacetyl-dihydro-azadirachtin, showed only one methoxyl absorption in its n.m.r. spectrum (fig. 15). The integration and appearance of the τ 8.0 - τ 8.5 region of the spectrum indicated that one of the acetate groups had been removed and the absorption due to the other had shifted upfield to become superimposed over the singlet of the tiglate ester absorption. There was no broad absorption in the infrared spectrum characteristic of the hydrogen bonded OH of a carboxylic acid group. This suggested that a lactone had been formed between the carboxyl group produced by the hydrolysis of a methyl ester and the hydroxyl group produced by the hydrolysis of the acetate group.

The expected molecular weight of such a derivative is 648, however the mass spectrum showed ions up to m/e 704, which could possibly be due to impurity, there was however no ion at 648 but a very intense ion at 649. Further work is required to correlate this compound with the structure of azadirachtin.

Azadirachtin was found to be very sensitive to acid, treatment with 2N. methanolic hydrochloric acid for two hours at room temperature and treatment with 0.1 N methanolic hydrochloric acid for three days at -5° , both gave a mixture of products which had very similar R_f values and could not be separated by preparative layer chromatography. The absorptions in the n.m.r. and infrared spectra due to the olefinic protons and double bond respectively of the dihydrofuran ring were no longer present in the spectra of the product. The appearance of an intense peak at τ 6.6 in the

60MHz. N.M.R. Spectrum of Deacetyl-dihydro-azadirachtin

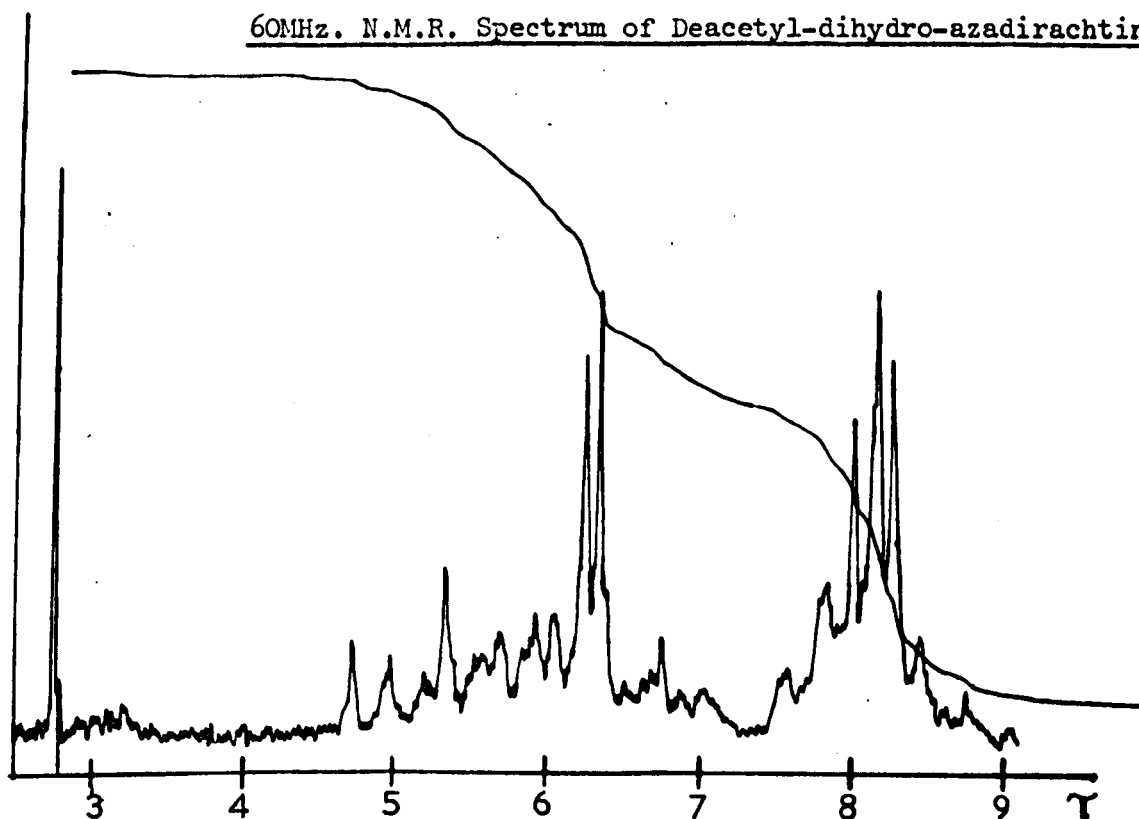


Figure 14

60MHz. N.M.R. Spectrum of the Major Product from the Alkaline Hydrolysis of
Dihydro-azadirachtin

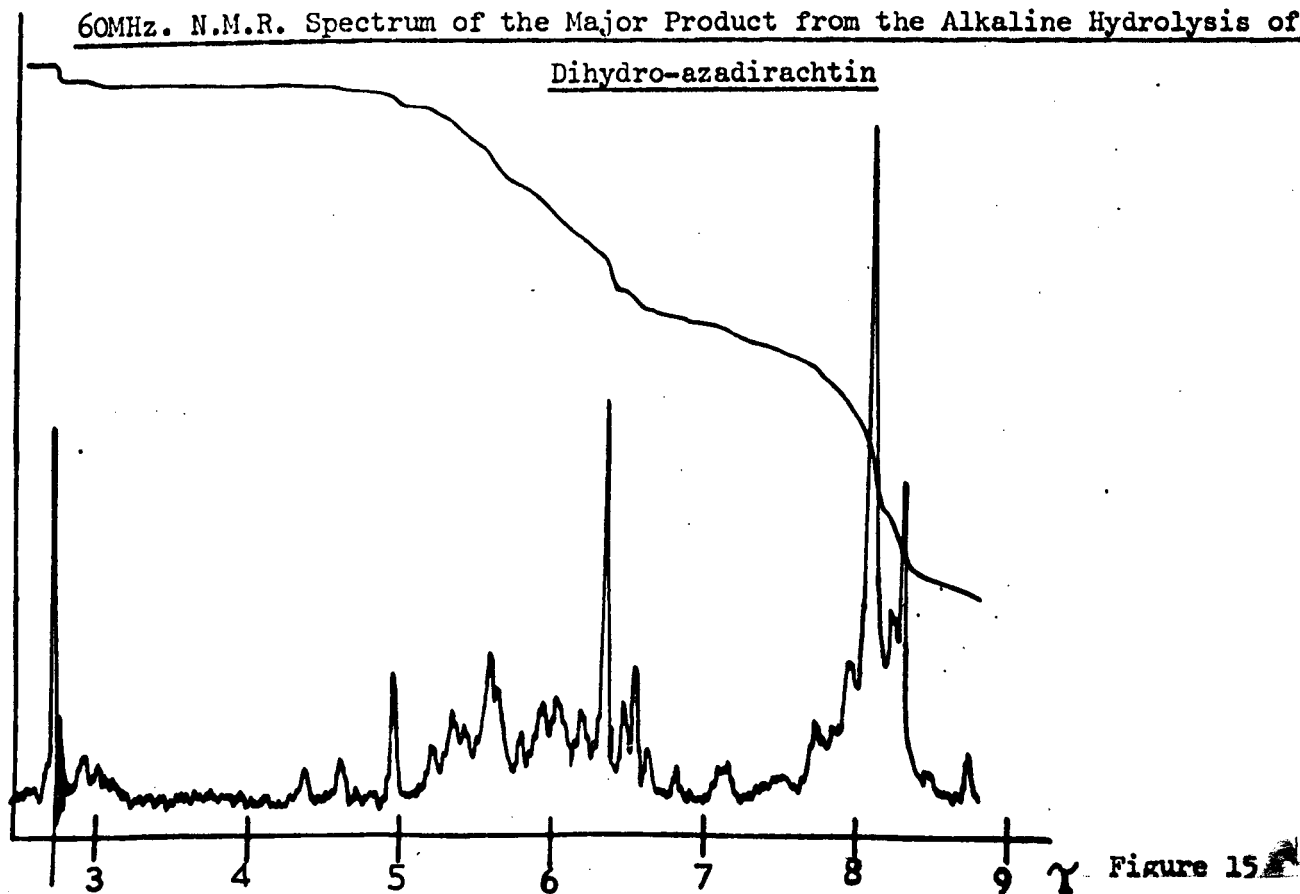


Figure 15

n.m.r. spectrum suggested that methoxyl groups had been introduced. It is possible that the acetal system already postulated in the structure of azadirachtin could have opened under the acid conditions with the formation of methyl acetals and other rearrangement products. There was no change in the relative intensities of the carbonyl absorption to that of the hydroxyl absorption in the infrared spectrum of the product compared to that of azadirachtin, indicating that no hydrolysis had occurred. Azadirachtin was found to be rather more stable to aqueous hydrochloric acid.

Dihydro-azadirachtin was also found to be sensitive to acid, treatment for one hour with 2N. methanolic hydrochloric acid was shown by thin layer chromatography to have given a mixture of at least three components.

Treatment of azadirachtin with dry hydrogen chloride in chloroform gave a product in which hydrogen chloride had added across the double bond of the dihydrofuran ring, this was deduced from the n.m.r. spectrum, which was very similar to that of azadirachtin except that the doublets assigned to the protons of the double bond of the dihydrofuran were no longer present, also the absorption due to this double bond in the infrared spectrum was absent.

Treatment of azadirachtin with bromine in chloroform gave a mixture of products the major one of which had an R_F on thin layer chromatography, similar to the products of acid treatment. When the reaction was repeated in the presence of suspended calcium carbonate, the product obtained had an R_F greater than that of azadirachtin. The n.m.r.

spectrum (fig. 16) of the product, purified by preparative layer chromatography, showed a shift of the doublet from $\tau 3.5$ upfield, indicating that the double bond of the dihydrofuran ring had been brominated, this was confirmed by the absence of an absorption at 1625 cm^{-1} in the infrared spectrum. The double bond of the tiglate ester group was not brominated, the characteristic absorptions of this group were still present in the n.m.r., infrared and ultraviolet spectra of the bromination product. The magnitude of the extinction coefficient in the ultraviolet spectrum was consistent with at least two atoms of bromine having been introduced into the molecule.

When lithium aluminium hydride reduction was carried out on azadirachtin a product was obtained which was extremely polar, being only soluble in alcohol, and which appeared to be a mixture of at least three components on thin layer chromatography. Such a reduction of ester groups would be expected to give rise to hydroxyl groups, that this had occurred was confirmed by a broad intense absorption at 3400 cm^{-1} and only a very weak absorption in the carbonyl region at 1720 cm^{-1} .

No further investigation of this reduction product was carried out, because its extreme polarity and general insolubility made this rather difficult.

As mentioned previously early attempts to prepare an acetate of azadirachtin were unsuccessful. Treatment of azadirachtin with pyridine and acetic anhydride for two days at room temperature gave no reaction. Treatment with warm acetic anhydride and treatment with pyridine-toluene-acetic anhydride both showed on thin layer chromatography the formation of

a major component with R_F on thin layer chromatography less than that of azadirachtin, with some minor components. This major component was not pursued at this stage because supplies of azadirachtin were limited and it was expected that an acetate of azadirachtin would be less polar than azadirachtin and hence have a greater R_F . Treatment of azadirachtin with acetic anhydride-boron trifluoride etherate gave a complex mixture of products.

At a later time, when more azadirachtin became available, the treatment with acetic anhydride was repeated on a larger scale. Azadirachtin was heated under reflux with acetic anhydride for ten minutes. The product which was isolated by evaporation of the acetic anhydride was found by thin layer chromatography to be a mixture of azadirachtin and another compound with lower R_F . The n.m.r. spectrum of this component at 60 MHz (fig. 17) and 100 MHz (fig. 18) clearly showed it to be a monoacetyl derivative of azadirachtin. The appearance of a single proton absorption at $\tau 4.8$ suggests that this is a proton on the carbon to which the new acetate group is attached, the absorption of this proton being in the complex absorption at $\tau 5.3$ in the spectrum of azadirachtin. The singlet at $\tau 4.4$ in the spectrum of azadirachtin which has been tentatively assigned to the proton of a hemi-acetal (27) or acetal (28) system is not shifted in the spectrum of the acetylated product. If a hemi-acetal system is present acetylation of the secondary hydroxyl group would be expected to occur readily, as in the case of melianone; it is clear that since the single proton absorption is not shifted, that such acetylation has not taken place. Therefore this tends to rule out the possibility of the hemi-acetal

60MHz. N.M.R. Spectrum of the Product from the Bromination of
Azadirachtin

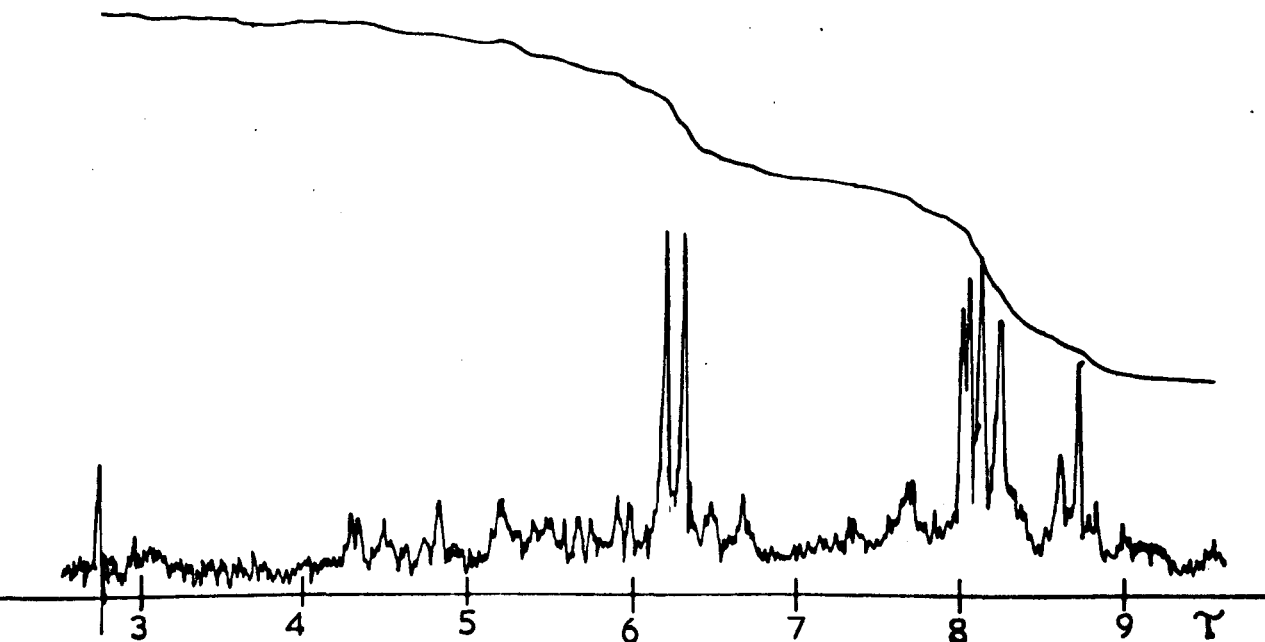


Figure 16

60MHz. N.M.R. Spectrum of Acetyl-azadirachtin

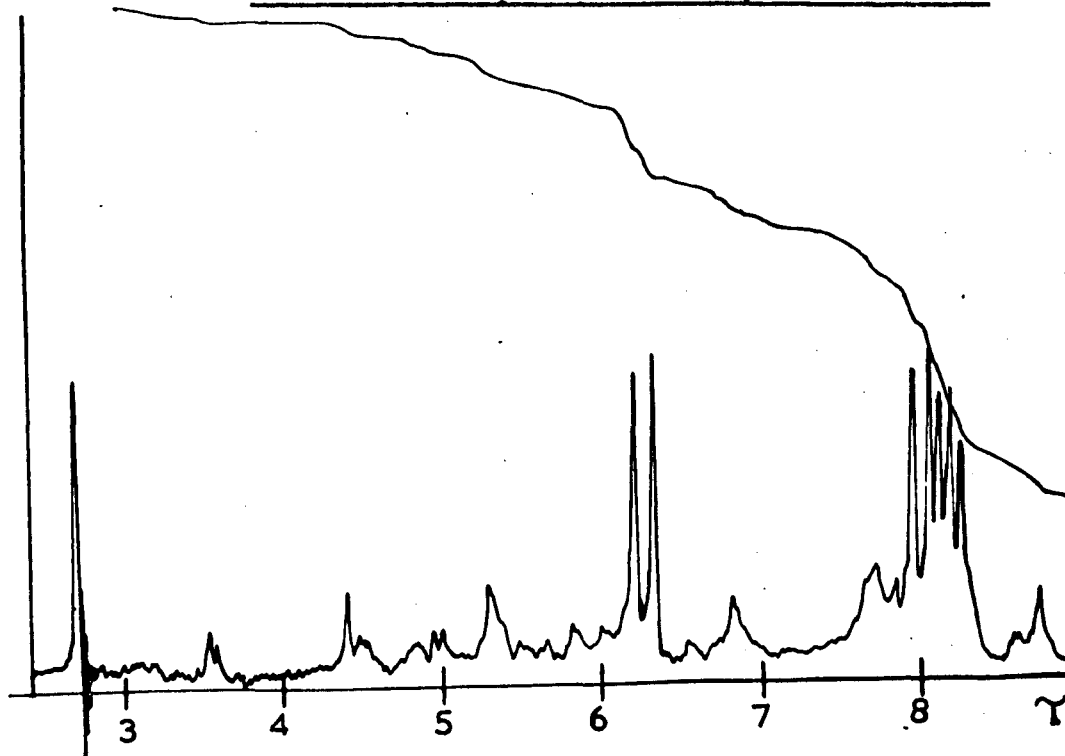


Figure 17

100MHz. N.M.R. Spectrum of Acetyl-azadirachtin

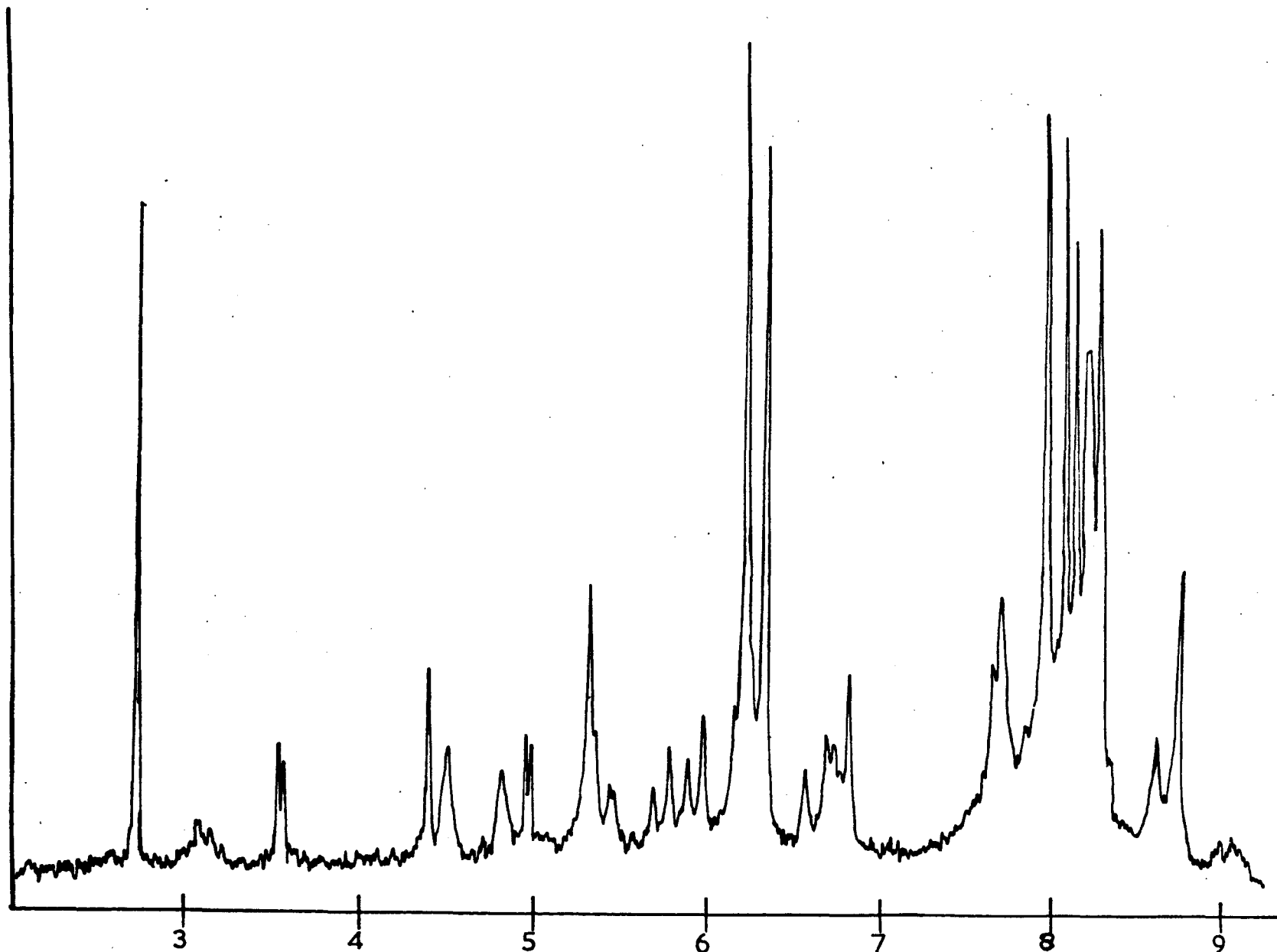
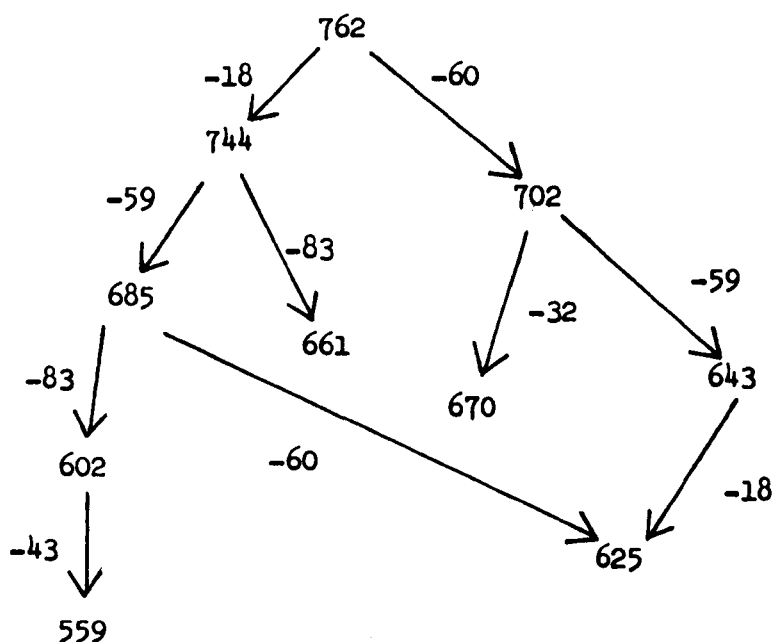


Figure 18

τ

system being present.

The expected molecular weight of acetyl-azadirachtin is 762, an ion of this mass is not observed in the mass spectrum; the ion of highest mass being m/e 744. All the ions above m/e 500 can be accounted for by loss of fragments which have already been identified in the structure. The loss of 83 mass units corresponds to the loss of C_5H_7O of the tiglate ester.



Although the total number of protons is known it is difficult to exactly determine how many protons are represented by each group of absorptions in the n.m.r. spectrum, only the single proton absorptions can be unambiguously recognised.

It has already been mentioned that some samples of azadirachtin showed a sharp absorption in their n.m.r. spectra at $\tau \sim 4.9$ which was exchangeable with D_2O , whereas with other samples this absorption was not

evident. It would appear from the integrated spectra of such samples, that the single proton doublet at $\tau 4.95$ is superimposed over a broad single proton absorption. It is probable that this absorption is due to the second hydroxyl group and that traces of acid in some of the samples cause the broad absorption to become sharp.

Spin decoupling of the n.m.r. spectrum of azadirachtin at 100 MHz (fig. 19) confirmed some of the assignments already made to the structure and also gave some additional information.

The coupling of the β -proton to the β -methyl protons ($J = \sim 10\text{Hz}$) of the tiglate ester group was shown by irradiation at $\tau 3.0$ and $\tau 8.25$. Confirmation that the doublets at $\tau 3.6$ and $\tau 4.95$ ($J = 3\text{Hz}$) were coupled to each other was obtained by irradiation at $\tau 4.95$.

Irradiation of the complex absorption at $\tau 5.3$ showed that it was coupled to the high field portion of the absorption at $\tau 7.7$ and to a doublet at $\tau 6.7$ ($J = 10\text{Hz}$), which varies in position with concentration. When the $\tau 6.7$ doublet was irradiated the extreme high field portion of the multiplet at $\tau 5.5$ coalesced with the rest of the multiplet.

On irradiation at $\tau 7.7$ the single proton absorption at $\tau 4.5$, which has been assigned to a proton on the carbon atom to which one of the acetate groups is attached, and the low field component of the $\tau 5.3$ multiplet became sharp singlets. This indicates that each of these absorptions is due to a proton on a carbon atom adjacent to a methylene group.

When the absorption at $\tau 4.5$ was irradiated a similar change was observed in the appearance of the absorption at $\tau 7.7$ as when the $\tau 5.3$ absorption was irradiated suggesting that the protons due to the

Spin-decoupling of the 100MHz. N.M.R. Spectrum of
Azadirachtin

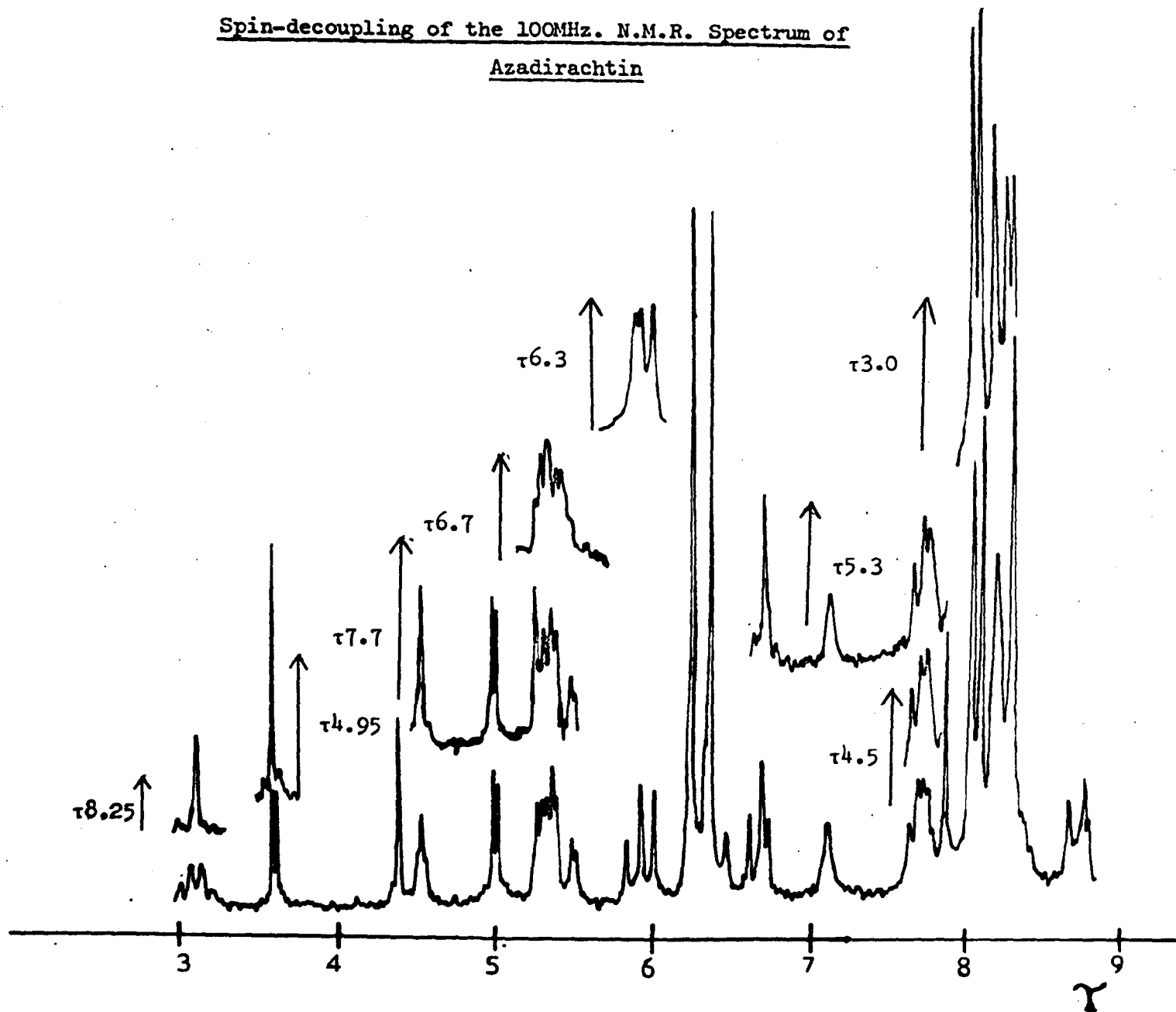
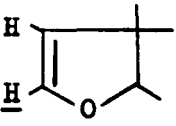
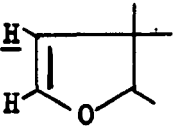


Figure 19

absorptions at τ 4.5 and τ 5.3 are coupled to the same methylene group. Such a system was put forward to explain the formation of an $\alpha\beta$ -unsaturated ketone when detigloyl-dihydro-azadirachtin was oxidized. This would mean that the proton absorbing at τ 5.3 is on the carbon atom to which the tigloyl group is attached.

The appearance of the two proton absorption at τ 5.9 in the 100 MHz (fig. 2) and 60 MHz spectra (fig. 1) suggested that it consists of two overlapping doublets ($J = 9$ Hz, in each case). This was clear from the 100 MHz spectrum of the acetate of azadirachtin (fig. 18) because here the doublets were completely separated. The low field doublet was found to be coupled to an absorption obscured by the methyl ester absorptions. No coupling was found for the high field doublet.

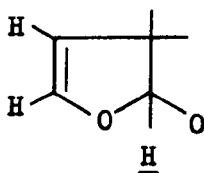
The following absorptions in the n.m.r. spectrum have been definitely assigned.

		No. of protons	
τ3.0	multiplet	$\begin{array}{c} \text{H} \\ \\ \text{C}=\text{C}-\text{C}(=\text{O})\text{O}- \\ \quad \quad \\ \text{H}_3\text{C} \quad \text{CH}_3 \end{array}$	1
τ3.6	doublet		1
τ4.55	unresolved triplet	$\text{AcO}-\text{CH}$	1
τ4.95	doublet		1
τ6.25	singlet	$\text{CH}_3\text{OC}(=\text{O})-$	3
τ6.35	singlet	$\text{CH}_3\text{OC}(=\text{O})-$	3
τ6.8-7.1	broad singlet	$-\text{OH}$	1
τ8.05	singlet	$\text{CH}_3\text{C}(=\text{O})\text{O}-$	3
τ8.1	singlet	$\text{CH}_3\text{C}(=\text{O})\text{O}-$	3
τ8.2	singlet	$\begin{array}{c} \text{H}_3\text{C} \quad \text{CH}_3 \\ \diagdown \quad \diagup \\ \text{C}=\text{C} \\ \diagup \quad \diagdown \\ \text{H} \quad \text{C}(=\text{O})\text{O}- \end{array}$	3

In addition to these definite assignments the following tentative assignments have been made.

$\tau 4.4$

singlet



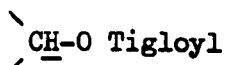
1

$\tau \sim 4.9$

-OH

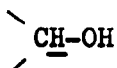
1

$\tau 5.3$



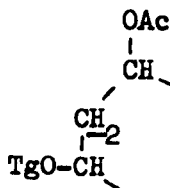
1

$\tau \sim 5.3$



1

$\tau 7.7$



2

Therefore a total of 15 protons remain to be accounted for, of these the absorptions between $\tau 5.0-7.0$ must be due to approximately eight protons on carbon atoms adjacent to oxygen atoms. It is not possible to say whether the singlet at $\tau 8.3$, obscured by the tiglate ester absorption represents two or three protons. If it is due to a methylene group the adjacent carbon atoms must be fully substituted,

because no coupling is observed. If it is due to a methyl group, the methyl group is considerably deshielded. Such deshielding would be possible if the methyl group were on a double bond, tetrasubstituted so that no coupling is observed. This double bond would have to be resistant to both oxidation and hydrogenation. Alternatively the deshielding could be caused by oxygen atoms adjacent to the carbon atom to which the methyl group is attached.

It is clear from the n.m.r. spectrum that azadirachtin does not bear any close resemblance to the limonoids and triterpenoids already isolated from neem, which are described in the Introduction. All of these compounds contain angular methyl groups which show absorptions between $\tau 8.5$ and $\tau 9.3$; the n.m.r. spectrum of azadirachtin does not show any singlet absorptions in this region.

Azadirachtin, $C_{35}H_{44}O_{16}$, is considerably more highly oxygenated than any of the triterpenoids and limonoids isolated from neem. It is possible that this compound may represent a further stage along the biogenetic pathway of increasing oxygenation.

The molecular formula, $C_{35}H_{44}O_{16}$, has fourteen double bond equivalents of which six are accounted for by the acetate, methyl ester and tiglate ester groups. The skeleton therefore has eight double bond equivalents, two are accounted for by the dihydrofuran ring, leaving six yet to be accounted for.

The structural features so far identified in azadirachtin account for the greater proportion of the atoms of the molecular formula, $C_{35}H_{44}O_{16}$. Since no close relationship appears to exist between

azadirachtin and the limonoids and triterpenoids of Meliaceae⁵³ and because this is a very complex molecule with many functional groups, much further work is necessary to arrive at the total structure.

Work is being carried out by Haskell and Mordue⁵⁴ on the physiological effect of azadirachtin on the desert locust (*Schistocerca gregaria*) and the site of the chemoreceptors which sense the molecule. In this work the level of activity of azadirachtin has been confirmed under similar experimental conditions.

EXPERIMENTAL

General Procedures

Pyridine was purified by distillation from potassium hydroxide pellets.

Melting points were determined on a Kofler hot stage apparatus.

Infrared absorption spectra were recorded on a Perkin Elmer 257 grating spectrophotometer.

Ultraviolet absorption spectra were recorded on a Unicam S.P.700 spectrophotometer using 1 cm. cells and commercial 95% ethanol as solvent.

Unless otherwise stated nuclear magnetic resonance (n.m.r.) spectra were recorded in deuteriochloroform on a Perkin Elmer R10 60 MHz instrument and are quoted as 'tau' (τ) values from an internal tetramethyl silane standard. Accumulated n.m.r. spectra were obtained using a Jeol JRA.1 spectrum accumulator in conjunction with the Perkin Elmer R10 instrument.

Unless otherwise stated mass spectra were determined on a Hitachi-Perkin Elmer RMU.6 instrument.

Chromatography

Benzene used in chromatography was dried over sodium and the ether used in chromatography was dried over calcium chloride.

Woelm alumina deactivated by the addition of water was used in column chromatography.

Thin layer chromatography (t.l.c.) was carried out on 5 x 20 cm. glass plates coated with silica gel G (Merck). The solvent was allowed to run 10 cm. up the plate. Components were visualised with iodine vapour.

Preparative thick layer chromatography (P.L.C.) was carried on 20 x 20 cm. and 40 x 20 cm. glass plates coated with a 1.5 mm. layer of Kieselgel PF₂₅₄ (Merck). The separated components, visualised under ultraviolet light, were isolated by scraping off the silica and then extracting it exhaustively with warm methanol either by eluting in a column or swirling with several portions of methanol in a flask. The filtered methanol solution was then evaporated, leaving a residue which contained some silica. The residue was dissolved in chloroform, the chloroform solution was filtered and evaporated.

Feeding Tests

Tests for antifeeding activity were carried out on mid-fifth instar hoppers of the desert locust (*Schistocerca gregaria*). Hoppers which had last been fed 24 hours previously were divided into groups of four insects in kilner jars arranged around a light source, each group of insects was presented with two 5.5 cm. Whatman No. 1 filter papers which had been impregnated with test solution allowed to dry and then sprayed with 0.25M sucrose solution and allowed to dry again. Each time a test was

carried out one group of insects was presented with filter paper impregnated with sucrose only, to act as a control. The test papers supported in corks were left with the insects for about eight hours. The results are quoted as the weight of test paper eaten expressed as a percentage of the weight of sucrose control paper eaten. The weight of sucrose control eaten was normally in the range of 100 - 300 mg.

The concentration of the test solutions of the extracts in the early part of the work are expressed in terms of the weight of seeds from which each fraction is derived.

Preliminary Examination of Neem Seeds for Antifeeding Activity

i) The Effect of Different Solvents in the Extraction of the Kernels

Kernels of neem seeds (17.5 g.) (from a Nigerian source) were extracted in turn with water (50 ml.), 95% ethanol (50 ml.) and light petroleum (b.p. 60-80°, 50 ml.), the resulting solutions were tested for locust antifeeding activity, at the original concentration and diluted to a tenth of the original concentration, none of the test papers were eaten.

ii) Extraction of the Shells

Shells of neem seeds were extracted with 95% ethanol in a Soxhlet apparatus, the resulting solution was found to be active in the feeding test.

iii) Volatility

Kernels of neem seeds (110 g. from an Indian source) were ground with water (300 ml.) in a mortar, the solution was filtered and evaporated at atmospheric pressure leaving a light brown powdery residue (8.7 g.) and a colourless distillate (250 ml.).

The kernels were further extracted in a Soxhlet apparatus with 95% ethanol (100 ml.). The ethanol solution was evaporated leaving a

dark brown sticky residue (3.5 g.) and a colourless distillate (60 ml.).

The kernels were then further extracted with light petroleum (b.p. 80-100°, 150 ml.) in a Soxhlet apparatus. The light petroleum was evaporated leaving a yellow-brown oil (12.6 g.) and a colourless distillate. The results of feeding tests on the residues and distillates are shown below.

Concentration of test soln.	Aqueous		Ethanol		Light Petroleum	
	res.	dist.	res.	dist.	res.	dist.
5 g. kernels/l	0%	34%	0%	169%		
3.3g. kernels/l	0%	36%	0%	80%	0%	192%
1 g. kernels/l	0%	190%	0%		60%	

iv) Extraction by Percolation

Kernels of neem seeds (95 g. from an Indian source) were ground and packed into a glass column filled with 95% ethanol. Ethanol was percolated through the column until extraction was complete. The green ethanol solution was evaporated leaving a dark brown oily residue (I, 6.9 g.)

The kernels from the ethanol extraction were ground with water in a mortar, the solution was filtered and evaporated leaving a light brown solid residue (5.2 g.)

Extraction of the kernels in a Soxhlet apparatus with 95% ethanol for four hours gave a brown solution which on evaporation yielded a brown sticky residue (1.7 g.).

Further extraction of the kernels with light petroleum (b.p. 80-100°) in a Soxhlet apparatus for a few hours, gave on evaporation of the light petroleum a yellow oil (14.7 g.).

v) Solvent Partition

Attempted partition of the ethanol extract (I) between chloroform and water and between n-butanol and water resulted in the formation of emulsions which could not be broken by the addition of methanol. However partition between light petroleum and methanol containing 5% of water resulted in the formation of two clear layers.

The ethanol extract (I, 6.9 g.) was dissolved in methanol containing 5% by volume of water and light petroleum (b.p. 80-100°, 50 ml.) giving a dark brown methanol layer and a green-yellow light petroleum layer. The layers were shaken together and allowed to separate. The methanol was evaporated leaving a sticky residue which on pumping (0.1 mmHg) became a light brown solid (II, 3.2 g.). The light petroleum was evaporated leaving a yellow oil (III, 1.8 g.).

The extracts (II) and (III) were tested for antifeeding activity with the following results.

	1 g. seeds/1	0.66 g. seeds/1	0.5 g. seeds/1
II	0%	0%	0%
III	0%	50%	142%

vi) Chromatography on Alumina

A column of alumina (activity III, 100 g.) was made up in light petroleum (b.p. 60-80°), the solid residue (II) was put onto the top of the alumina and the column was eluted as shown in the table below, the fractions eluted were arbitrarily divided into 11 groups.

Group	Fractions (100 ml.)	Range of Eluting Solvents	Weight g.
	1	Light petroleum (b.p. 60-80°)	0
1	2 - 8	Light petroleum: benzene (9:1) - benzene	0.121
2	9 - 20	Benzene: ether (9:1) - ether	0.272
3	21	Ether: methanol (9:1)	0.007
4	22 - 23	Ether: methanol (4:1)	0.024
5	24 - 28	Ether: methanol (7:3) - ether: methanol (2:3)	0.019
6	29 - 31	Ether: methanol (3:7) - ether: methanol (1:9)	0.033
7	32 - 35	Methanol	0.068
8	36 - 38	Methanol	0.149
9	39 - 44	Methanol	0.302
10	44 - 54	Methanol	0.058
11	54 - 61	Methanol	0.067

The groups of fractions were tested for anti-feeding activity, the results are shown below.

Eluent group.	Concentration g.seeds/l			
	6.6	3.3	1.7	1.1
1	112%	76%		
2		0%	19%	0%
3		0%	13%	0%
4	0%	60%		0%
5		0%	0%	0%
6		0%	0%	63%
7		0%	0%	
8		0%	50%	
9		4%	100%	
10		177%		
11		60%		

The Standard Extraction Procedure

Whole neem seeds (500 g.) were ground with 95% ethanol (625 ml.) in a Waring blender, the residue of the seeds was filtered off and treated with a further quantity of ethanol (500 ml.) in the blender. The yellow-green solution was evaporated leaving an oily green residue containing some brown solid. This residue was partitioned between aqueous methanol (5:95, 150 ml.) and light petroleum (b.p. 40-60°, 150 ml.). The two layers were separated and the light petroleum was extracted with more aqueous methanol (50 ml.). The combined methanol extracts were evaporated leaving a brown sticky residue (IV, 17.8g.). The light petroleum was evaporated leaving a yellow-green oil (14.3 g.) which was discarded.

Experiments Directed to the Isolation of the Antifeeding Component

i) Chromatography on Alumina

A portion of the methanol partition residue (IV, 5 g.) was redissolved in methanol (30 ml.), to this was added alumina (5 g.) and the methanol was evaporated leaving a lumpy residue. A small amount of Kieselgel was added and the mixture was mixed to a smooth paste with methanol, this was then evaporated (0.1 mmHg.) leaving a powdery residue.

This residue was put onto a column of alumina (activity III, 100 g.) made up in light petroleum and the column was eluted as shown in the table below. A portion of each fraction (500 ml.) eluted was diluted 75 times and tested for antifeeding activity.

Fraction	Eluting Solvent	Feeding Test
1	Benzene	98%
2	Benzene-methanol (9:1)	0%
3	" " (4:1)	58%
4	" " (7:3)	80%
5	" " (3:2)	125%
6	" " (1:1)	106%
7	" " (2:3)	44%
8	" " (3:7)	29%
9	" " (1:4)	93%
10	" " (1:9)	74%
11	Methanol	81%
12	Methanol	83%

Fraction 2 was evaporated leaving a light yellow glassy residue (V, 1.5 g.), a portion of this residue (1.0 g.) was chromatographed on a column of alumina (activity III, 25 g.). The table below shows the fractions collected with the results of the tests for antifeeding activity.

Fraction	Eluting solvent (100 ml.)	Weight of fraction g.	Each fraction dissolved in 50 ml. methanol and diluted:		
			20 times	200 times	500 times
1	Benzene	0.199	102%	189%	69%
2	Benzene-ether (9:1)	0.215	72%	85%	49%
3	" " (4:1)	0.077	75%	137%	63%
4	" " (7:3)	0.077	37%	50%	84%
5	" " (3:2)	0.080	0%	34%	34%
6	" " (1:1)	0.062	0%	17%	103%
7	Ether	0.037	0%	0%	83%
8	Ether-methanol (9:1)	0.097	0%	0%	0%
9	" " (4:1)	0.044	0%	65%	101%
10	" " (7:3)	0.029	0%	135%	82%
11	" " (3:2)	0.015	20%		112%

ii) Large Scale Chromatography on Alumina

Neem seeds (880 g.) were extracted as described in the Standard Extraction Procedure. The sticky brown solid (34 g.), obtained from the methanol phase, was absorbed onto Kieselgel (28 g.) and alumina in the way described in the previous section, this was then put onto the top of a column of alumina (activity III, 550 g.) made up in benzene and the column was eluted exhaustively with benzene-methanol (9:1) (2250 ml.). Evaporation of the eluent gave a yellow solid (VI, 11.5 g.).

A portion of this residue (VI, 5 g.) was further chromatographed on a column of alumina (activity III, 100 g.). The fractions obtained and

the results of feeding tests are shown in the table below.

Fraction	Eluting solvent (500 ml.)	Weight of fraction g.	Each fraction dissolved in 50 ml. methanol diluted:		
			100 times	1,000 times	10,000 times
1	Benzene	1.87			
2	Benzene-ether (9:1)	0.55			
3	" " (4:1)	0.55	180%		
4	" " (7:3)	0.25	109%		
5	" " (3:2)	0.20	70%		
6	" " (1:1)	0.12	75%		
7	Ether	0.01	162%		
8	Ether-methanol (9:1)	0.25	0%	0%	73%
9	" " (4:1)	0.13	0%	73%	52%
10	" " (7:3)	0.11	56%		
11	" " (3:2)	0.01			
12	" " (1:1)	0.01			
13	Methanol	0.01			

iii) Isolation of Nimbin

Crystallization of fraction 1 from 95% ethanol gave nimbin as a colourless microcrystalline solid m.p. 178-180° (0.35 g.). Three successive recrystallizations from ether raised the m.p. to 198-201° (lit.¹⁵ m.p. 205°). The infrared spectrum was identical with that of a sample of nimbin provided by Dr. Narasimhan (Found: C 66.7; H 7.03. Calc. for C₃₀H₃₆O₉: C 66.7; H 6.7). The sample on t.l.c. in benzene-acetone (4:1) showed an

intense spot R_F 0.49 with a faint spot R_F 0.34, the sample provided by Dr. Narasimhan showed an intense spot R_F 0.49 with two faint spots R_F 0.34 and R_F 0.20.

iv) Chromatography of the Ether-methanol (9:1) Fraction on Florisil

The ether-methanol (9:1) fraction (fraction 8 above) was chromatographed on a column of florisil (100-200 mesh, 10 g.) eluting successively with benzene, benzene-ether mixtures, ether, ether-ethyl acetate mixtures, ethyl acetate, ethyl acetate-methanol mixtures and methanol. The fractions collected were evaporated and dissolved in methanol (50 ml.), a portion of each solution was diluted 50 times with methanol and tested for antifeeding activity. All the fractions eluted by ether-ethyl acetate mixtures were found to possess activity.

The ether-ethyl acetate fractions were combined (62 mg.) and subjected to P.L.C. eluting six times with ether-acetic acid-acetone (98:1:1). Three bands were separated, these gave colourless brittle solids, (VII) R_F 0.22 (2.0 mg.) (VIII) R_F 0.18 (2.7 mg.) and (IX) R_F 0.10 (0.2 mg.) on t.l.c. in ether-acetic acid acetone (98:1:1).

Each of these fractions was dissolved in chloroform (5 ml.) and a portion of each solution was tested for antifeeding activity after diluting with methanol, the results are shown below.

Chloroform solution diluted with methanol						
	100 times	500 times	1000 times	2000 times	4000 times	10,000 times
VII	0%	0%	0%	2%	119%	76%
VIII	0%	0%	29%	114%		
IX		103%				

v) Isolation of Azadirachtin

The remainder of the benzene-methanol (9:1) fraction obtained above was chromatographed on a column of alumina (activity III, 120 g.) as before, the fractions collected are shown in the table below.

Fraction	Eluting solvent (500 ml.)	Weight g.
1	Benzene-ether (1:1)	3.607
2	Ether	0.624
3	Ether-methanol (9:1)	0.407
4	" " (4:1)	0.204
5	" " (7:3)	0.086
6	" " (3:2)	0.128
7	Methanol	0.047

Fractions 2, 3 and 4 all showed on t.l.c. components with R_F

values in the region of the active material (VII), fraction 3 appeared to have this component in the greatest proportion. Fractions 2, 3 and 4 were each dissolved in methanol (10 ml.) and portions of these solutions diluted 250 times were tested for antifeeding activity, all were active.

P.L.C. of fraction 3 (300 mg.) eluting six times with ether-acetic acid-acetone (98:1:1) gave three bands with an intense band at the origin and considerable streaking between the bands.

The upper band gave a colourless brittle solid (X 35 mg.) m.p. 148-158°. $\lambda_{\text{max.}}$ (EtOH) 218 nm. $E_{1\text{ cm.}}^{1\%}$ 142. $\nu_{\text{max.}}$ (KBr. disc) 3460 (broad) 1740 (strong), 1710 (shoulder), 1650 (weak) and 1620 cm^{-1} (weak). $\nu_{\text{max.}}$ (CCl_4) 3460 (broad), 1745 (strong), 1720 (shoulder), 1710 (shoulder), 1655 (weak) and 1625 cm^{-1} (weak). This material on t.l.c. eluting three times with chloroform-ethyl acetate (1:1) showed a single component R 0.25 identical with fraction (VIII) run on the same plate. The name *azadirachtin* was given to this compound.

The middle band gave a colourless brittle solid (XI, 20 mg.) m.p. 148-151°. $\lambda_{\text{max.}}$ (EtOH) 213 nm. $E_{1\text{ cm.}}^{1\%}$ 61. $\nu_{\text{max.}}$ (KBr disc) 3470 (broad), 1730 (strong) 1650 (weak) and 1620 cm^{-1} (weak). This material was found to be inhomogeneous, on t.l.c. eluting three times with chloroform-ethyl acetate (1:1) the fraction showed two components the major one R 0.20 and the minor R 0.25 corresponding to azadirachtin.

Solutions of 1 mg. of azadirachtin (X) in 10 ml. of chloroform and of 6.0 mg. of the middle component (XI) in 10 ml. of chloroform were

each diluted 250 and 500 times with methanol; these solutions were tested for antifeeding activity, all of them showed activity.

Modified Procedure for the Isolation of Azadirachtin

i) Methylene Chloride-Water Partition

Neem seeds (1000 g.) were extracted using the Standard Extraction Procedure. The sticky brown residue (39 g.) from the methanol phase was partitioned between water (400 ml.) and methylene chloride (400 ml.), the layers were shaken together and the aqueous layer became emulsified. The clear methylene chloride layer was separated and the aqueous layer was shaken with more methylene chloride (100 ml.) and left for two hours, the aqueous layer remained emulsified. The clear methylene chloride layer was separated and the combined methylene chloride extracts were evaporated to a smaller volume (~ 100 ml.). The residue obtained on evaporation of the aqueous layer on t.l.c. in ether-acetic acid-acetone (98:1:1) showed an intense spot at the origin with a faint streak to the solvent front. The methylene chloride solution was poured into light petroleum (b.p. 60-80°, 500 ml.) with stirring, a yellow precipitate was formed which was filtered off (XII, 18.3 g.). Evaporation of the light petroleum gave a yellow oil which on t.l.c. in ether-acetic acid-acetone (98:1:1) showed only a faint streak from the origin with most of the material between $R_f \sim 0.5$ and the solvent front, whereas the precipitate showed a component R_f 0.2 corresponding to

azadirachtin with a number of less polar components.

ii) Chromatography

Chromatography of the precipitated solid (XI 5.3 g.) on alumina (activity III, 100 g.) eluting with benzene, benzene-ether, ether and ether-acetone was shown to have achieved no efficient separation, so the fractions were recombined (2.0 g.) and chromatographed on a column of B.D.H. silica gel (60 g.), the fraction (1.2 g.) eluted by benzene (300 ml.) contained material in the range of R_F of azadirachtin and was further chromatographed on a column of florisil (100-200 mesh, 22 g.) and the following fractions were collected.

Fraction (50 ml.)	Eluting Solvent	Weight mg.
1-5	Benzene	27
6-21	Benzene-ether (9:1)	251
22-33	Benzene-ether (4:1)	120
34-44	Benzene-ether (7:3)	152
45-47	Benzene-ether (3:2)	7
48-49	Benzene-ether (1:1)	1
50-55	Ether	25
56-63	Ether-acetone (9:1)	86

The fractions were subjected to t.l.c. using ether-acetic acid-acetone (98:1:1) as the development solvent, fractions 10 to 50 showed components R_F 0.3 and R_F 0.24 (azadirachtin) the later fractions

contained the greater proportion of azadirachtin.

P.L.C. of fractions 30 to 50 (142 mg.) eluting twice with ether-acetic acid-acetone (98:1:1) gave two bands. The lower more intense band gave a colourless residue (XIII, 55 mg.) shown to be identical with azadirachtin by infrared spectroscopy and t.l.c.

R_F 0.24 in ether-acetic acid-acetone (98:1:1) R_F 0.15 in ether-acetone (49:1) . The less intense upper band gave a colourless glassy material (XIV, 10 mg.) R_F 0.3 in ether-acetic acid-acetone (98:1:1). The two components were tested for antifeeding activity as shown below.

0.35 mg. of component dissolved in 100 ml. methanol diluted:			
	0 times	10 times	50 times
XIII (azadirachtin)	0%	0%	0%
XIV	35%	54%	

Standard Procedure for the Isolation of Azadirachtin

Neem seeds (2 kg.) were ground with 95% ethanol (2.5 l.) in a Waring blender. The solution was filtered and the residue of the seeds was ground with more 95% ethanol (2 l.) and the solution was filtered. The combined ethanol extracts were evaporated leaving an oily residue (170 g.) which was partitioned between light petroleum (b.p. 40-60°, 800 ml.

and aqueous methanol (5:95, 800 ml.). The layers were separated and the light petroleum was washed with more aqueous methanol (5:95, 200 ml.). The combined methanol extracts were evaporated leaving a dark brown sticky residue (76 g.), this was treated with warm toluene (\sim 300 ml.) and the resulting dark brown solution was chromatographed on a column of Floridin earth (BDH Florex XXS, 950 g.), made up in toluene, eluting with ether-acetone (95:5). The following fractions were collected:

Fraction (200 ml.)	Weight g.	Fraction (200 ml.)	Weight g.
1	9.63	14	0.30
2	6.58	15	0.27
3	3.69	16	0.37
4	3.10	17	0.24
5	2.79	18	0.37
6	2.85	19	0.30
7	1.59	20	0.31
8	0.87	21	0.18
9	0.82	22	0.19
10	0.46	23	0.12
11	0.41	24	0.11
12	0.35	25	0.11
13	0.23	26	0.09

On t.l.c. in ether-acetone (4:1) fractions 1-9 showed components at R_F 0.73, 0.65, 0.56 and 0.50. Fractions 10-14 showed the major component

at R_F 0.45, fraction 14 appeared to contain some azadirachtin R_F 0.42. Fractions 15-25 contained azadirachtin R_F 0.42 as the major component, this was confirmed by n.m.r. spectroscopy.

Fractions 15-25 were combined (2.57 g.) and subjected to P.L.C. on ten 40 x 20 cm. plates eluting twice with ether-acetone (95:5). The intense band 5 cm. from the origin gave azadirachtin (1.51 g.) m.p. 149-153°.

Azadirachtin

The R_F values of azadirachtin in various solvents are listed below.

	R_F
Ether-acetone-acetic acid (98:1:1)	0.22
Ether-acetone (49:1)	0.15
Ether-acetone (4:1)	0.50
Ethyl acetate	0.60
Chloroform-acetone (7:3)	0.40

Azadirachtin was insoluble in light petroleum, virtually insoluble in ether, slightly soluble in benzene and carbon tetrachloride and soluble in chloroform, methanol, ethanol and acetone. Attempts to crystallize azadirachtin were not very successful, with carbon tetrachloride

it was possible to crystallize azadirachtin as a fine powder
m.p. 154-158°.

Infrared Spectrum

$\nu_{\text{max.}}$ (CCl₄) 3460 cm⁻¹ (broad), 1745 (strong), 1720 (shoulder),
1710 (shoulder), 1655 (weak) and 1625 cm⁻¹ (weak).

Ultraviolet Spectrum

$\lambda_{\text{max.}}$ 217 nm. $\epsilon_{\text{max.}}$ 9200 (assuming M.W. 720)

N.M.R. Spectrum

The n.m.r. spectrum was recorded at 60 MHz and 100 MHz. These
are shown in fig. 1 and fig. 2.

Mass Spectrum

The mass spectrum was first recorded on the M.S. 9 instrument
at the Shell Laboratory for Chemical Enzymology. The highest peak of
any significance was at 642, the accurate mass of this peak was measured and
the molecular formula C₂₉H₃₈O₁₆ was assigned. However an error was
discovered and the molecular formula was corrected to C₃₃H₃₈O₁₃, also a
number of other significant peaks were measured accurately.

m/e	Measured Mass	Formula
642	642.229824	$C_{33}H_{38}O_{13}$
627	627.208563	$C_{32}H_{35}O_{13}$
611	611.211973	$C_{32}H_{35}O_{12}$
560	560.222681	$C_{29}H_{36}O_{11}$
560	560.189327	$C_{28}H_{32}O_{12}$
559	559.217213	$C_{39}H_{35}O_{11}$
527	527.191153	$C_{28}H_{31}O_{10}$
475	475.160267	$C_{24}H_{27}O_{10}$
233	233.080891	$C_{13}H_{13}O_4$
131	131.049647	C_9H_7O
131	131.085669	$C_{10}H_{11}$
105	105.033788	C_7H_5O
105	105.069386	C_8H_9
100	100.052606	$C_5H_8O_2$
83	83.049654	C_5H_7O

The mass spectrum of azadirachtin was recorded on a Hitachi-Perkin Elmer RMU.6 instrument, this showed some change over the spectrum recorded previously, the m/e 500-700 region of the spectrum is shown in fig. 3.

Optical Rotation

$$[\alpha]_D -53^\circ [c.0.5 (CHCl_3)]$$

Analysis

Repeated analysis of azadirachtin did not give consistent results. A sample of azadirachtin recrystallized from carbon tetrachloride and dried by pumping on an oil pump at 0.1 mmHg and 100° for several hours, was found to still contain some carbon tetrachloride when the mass spectrum was recorded. Carbon-hydrogen analysis gave C, 56.9; H, 6.00%.

Analysis of a sample purified by P.L.C. gave C, 58.8; H, 6.54%.

$C_{35}H_{44}O_{16}$ requires C, 58.3; H, 6.1%.

Antifeeding Activity

Three separate tests were carried out at low concentration with the following results.

	Test Number		
	1	2	3
<hr/>			
Conc ⁿ . of test sol ⁿ mg./l.			
0.11	0%		
0.08		0%	0%
0.055	0%		
0.04		0%	27%
<hr/>			

Alkaline Hydrolysis of Azadirachtin to give Tiglic Acid

Azadirachtin (30 mg.) was dissolved in methanol (1 ml.) and 0.3N sodium hydroxide (1 ml.). The solution was refluxed for 30 minutes giving a light brown solution, which was cooled in an acetone-dry ice bath and pumped at 0.1 mmHg. for four hours. The residue was acidified with 1N. hydrochloric acid (0.33 ml.). A small volume of the aqueous solution was treated with ethereal diazomethane, prepared by treating N-nitrosomethylurea with base. The ether solution was subjected to gas liquid chromatography (g.l.c.) on a 5 ft. long $\frac{1}{8}$ in. diameter column of 10% polyethylene glycol adipate on D.M.C.S. treated support "M" using a flow rate of 50 ml./min.

At 101° the following peaks were recorded.

Relative Peak Heights	
0.7 min.	off scale
1.2 min.	off scale
1.5 min.	100%
2.0 min.	8.9%
3.9 min.	2.2%
4.2 min.	89%

Methyl tiglate prepared by treating tiglic acid with ethereal diazomethane showed on g.l.c. under the same conditions an intense peak at 4.2 min. with two weaker peaks at 1.3 and 1.5 min.

A solution of diazomethane in ether was shaken with dilute

hydrochloric acid, the ether solution was subjected to g.l.c. under the same conditions as before, apart from the solvent peak, peaks were observed at 1.3 and 1.5 min.

Hydrogenation of Azadirachtin

i) At Atmospheric Pressure

Adam's platinum catalyst (3 mg.) in glacial acetic acid (9 ml.) was reduced by hydrogen in a microhydrogenator, when the uptake of hydrogen had ceased azadirachtin (15 mg.) was added and the mixture was shaken for two hours, no further uptake of hydrogen occurred. The solution was filtered and evaporated leaving a light brown residue (11 mg.), shown to be unchanged azadirachtin by t.l.c. and its infrared spectrum.

ii) At Increased Pressure

Adam's platinum catalyst (20 mg.), azadirachtin (50 mg.) and ethyl acetate (50 ml.) were shaken with hydrogen at a pressure of 50 lbs./sq.in. for one hour. The solution was filtered and evaporated. The residue showed on t.l.c. in ether-acetone (4:1) a component R_F 0.25 with some azadirachtin R_F 0.48. P.L.C. gave the major component *dihydro-azadirachtin* as a colourless glass (30 mg.) R_F 0.25 in ether-acetone (4:1) m.p. 119-122° $[\alpha]_D -15^\circ$ $[c\ 0.4\ (CHCl_3)]$ λ_{max} 219 nm. ϵ_{max} 7300 (assuming M.W. 722) ν_{max} (CCl₄) 3450 (broad) 1740 (strong), 1710 (strong) and 1650 cm⁻¹ (weak).

The n.m.r. and mass spectra are shown in figs. 5 and 4.

Preparation of the Trimethylsilyl Ether Derivative of Azadirachtin

Azadirachtin (80 mg.) was dissolved in chloroform (1 ml.) bis-(trimethylsilyl) acetamide (0.5 ml.) was added and the mixture was left for ten minutes. The volatile components were evaporated (0.1 mmHg.) leaving a gummy brown residue which on t.l.c. in ether-acetone (9:1) showed an intense spot R_F 0.66, a faint spot R_F 0.50 and some azadirachtin R_F 0.27.

The residue was subjected to P.L.C. eluting once with ether-acetone (9:1). The major band was scraped off and the silica was extracted with chloroform, the chloroform was evaporated leaving *bis-trimethylsilyl-azadirachtin* as a colourless glassy residue (42 mg.), m.p. 110-112°.

Found: C, 57.3; H, 7.4% $C_{41}H_{60}O_{16}Si_2$ requires C, 56.9; H, 7.0%.

ν_{max} . (CCl_4) 3570 (weak), 1750 (strong), 1710 (strong), 1655 (weak) and 1620 cm^{-1} (weak). The n.m.r. spectrum is shown in fig. 6. The mass spectrum above m/e 600 is shown in fig. 7.

Accurate mass determination of the molecular ion carried out by Dr. Williams at the Shell Laboratory for Chemical Enzymology Sittingbourne and by the Physico-chemical Measurements Unit, Harwell, gave 864.3454 and 864.3438 respectively, errors of 4 p.p.m. and 2 p.p.m. from 864.3420 the calculated mass for $C_{41}H_{60}O_{16}Si_2$.

Attempted Oxidation of Azadirachtin with Potassium Permanganate

Azadirachtin (10 mg.) was dissolved in acetone (20 ml.) which

had been refluxed over potassium permanganate for one hour and then distilled. The solution of azadirachtin was refluxed on a steam bath and a solution of potassium permanganate in acetone (1 g./l.) was added drop by drop until the permanganate colour persisted (~ 20 ml.). The solution was concentrated by evaporation and a solution of sodium bisulphite in dilute hydrochloric acid was added, the solution became colourless; this solution was then extracted with ethyl acetate, which was then washed with water and evaporated leaving a yellow residue. The residue on t.l.c. in ether-acetone (4:1) showed two elongated spots at R_F 0.06 and R_F 0.15, with streaking to R_F 0.6. The residue was esterified by dissolving in chloroform and treating with ethereal diazomethane. The product on t.l.c. in ether-acetone (4:1) showed no definite spots but a streak from the origin to the solvent front.

Ozonolysis of Azadirachtin

Ozonized air was bubbled through a solution of azadirachtin (78 mg.) in ethyl acetate (50 ml.) at a temperature between -20 and -30° for five minutes. Adam's platinum catalyst (30 mg.) was added to the solution which was then stirred in an atmosphere of hydrogen at 0° until no more hydrogen was taken up. The solution was filtered and evaporated leaving a colourless residue (79 mg.) which on t.l.c. in ether-acetone (4:1) showed a streak from the origin to R_F 0.2. The n.m.r. spectrum was recorded and is shown in fig. 8. The residue, which had an odour resembling

that of a low molecular weight carboxylic acid, was pumped at 0.1 mmHg. for ten hours. Now the residue on t.l.c. no longer appeared streaked but showed components at R_F 0.08, R_F 0.15 and R_F 0.24. The residue was subjected to P.L.C. eluting with ether-acetone (95:5), however no distinct bands were visible, the product appeared as a streak from the origin to R_F 0.25.

Oxidation of Azadirachtin with the Lemieux Reagent (Sodium Metaperiodate-Potassium Permanganate)

Azadirachtin (57 mg.) was dissolved in t-butanol (20 ml.) and water (30 ml.) and the pH was adjusted to 7.7 with sodium carbonate. A solution of 98.33 mM. sodium metaperiodate and 1.67 mM. potassium permanganate (20 ml.) was added and the mixture was left at room temperature for one hour. The solution was then acidified with dilute hydrochloric acid and extracted with chloroform. The chloroform was washed with water and evaporated leaving a colourless residue (0.61 g.) ν_{\max} . (CHCl_3) 3450 (broad), 3500-2500 (very broad), 1730 cm^{-1} (strong). The n.m.r. spectrum is shown in fig. 9. On t.l.c. in ether-acetone (4:1) the product showed an elongated spot at the origin characteristic of a carboxylic acid.

This residue was dissolved in chloroform and treated with ethereal diazomethane at 0° , the solvent was evaporated leaving a colourless glassy residue which was not homogeneous, on t.l.c. in ether-acetone (4:1) it showed spots R_F 0.40 and 0.56 with streaking; in chloroform-acetone (1:1) it showed a diffuse spot R_F 0.52 and in ethyl acetate it showed streaking

with spots at R_F 0.6 and 0.76. ν_{\max} . (CHCl_3) 3420 (broad), 1730 (strong), and 1620 cm^{-1} (weak). The n.m.r. spectrum showed an increase in absorption at $\tau 6.3$ and $\tau 8.4$ as compared to the spectrum of the oxidation product. There was no absorption at $\tau 7.5$ but the $\tau 2.2$ absorption was still present.

Hydrolysis of the Lemieux Oxidation Product of Azadirachtin

The Lemieux oxidation product (146 mg.) was dissolved in methanol (18 ml.) and an aqueous solution of potassium bicarbonate (450 mg. in 9 ml.) was added. After 24 hours the solution was acidified and extracted with chloroform, the chloroform was washed with water and evaporated leaving a residue which was esterified with ethereal diazomethane. The product on t.l.c. in ether-methanol (9:1) showed three components R_F 0.2, R_F 0.29 and R_F 0.55 with considerable streaking between. The mixture was subjected to P.L.C. eluting three times with ether-methanol (49:1), three components were isolated. The middle component was a colourless glass (16 mg.), on t.l.c. eluting three times with ether-methanol (49:1) R 0.35. Its n.m.r. spectrum showed an intense absorption at $\tau 6.3$ in the methyl ester region, one acetate group had apparently been lost and there was an absorption at $\tau 8.7$. The upper component (9 mg.) R 0.40 eluting three times with ether-methanol (49:1) and lower component (3 mg.) R 0.30 in the same solvent system both had n.m.r. spectra very similar to that of the major component.

Attempted Oxidation of Azadirachtin with Cornforth's Reagent
(Chromium Trioxide-Pyridine)

Cornforth's Reagent was prepared by adding a solution of chromium trioxide (5 g.) in water (3 ml.) to pyridine (50 ml.) cooled in ice.

Azadirachtin (10 mg.) was dissolved in pyridine (0.05 ml.) and Cornforth's reagent (0.25 ml.) was added. After two days water was added and the solution was extracted with chloroform, the chloroform was washed with water and evaporated. The residue (6 mg.) on t.l.c. in ether-acetone (4:1) showed a spot corresponding to azadirachtin and an intense spot at the origin, corresponding to impurity of the reagent.

Oxidation of Dihydro-azadirachtin with the Lemieux Reagent
(Sodium Metaperiodate-Potassium Permanganate)

Dihydro-azadirachtin (98 mg.) was dissolved in t-butanol (30 ml.) and water (40 ml.); sodium carbonate was added to give a pH of about 7.7 followed by a solution of 98.33 mM sodium metaperiodate and 1.67 mM potassium permanganate (30 ml.). The mixture was left for 75 minutes and extracted with chloroform. The chloroform was washed with water and evaporated leaving a colourless glassy residue (0.090 g.) which on t.l.c. in ether-acetone (4:1) showed a major component R_F 0.19 with some starting material R_F 0.29.

This residue was subjected to P.L.C. eluting twice with ether-acetone (4:1). *Detigloyl-pyruvyl-dihydro-azadirachtin* was isolated as a

colourless solid (21 mg.) m.p. 147-150°. R_F Ether-acetone (4:1) 0.19
 ν_{\max} . 3440 (broad), 1740 (strong), 1720 cm^{-1} (shoulder) λ_{\max} . 227 nm.
 ϵ_{\max} . 1500. The n.m.r. spectrum is shown in fig. 10. The mass spectrum
showed significant ions beyond m/e 500 at 692 (1), 660 (7), 649 (14),
632 (13), 617 (31), 605 (10) 589 (40), 575 (26), 561 (16), 547 (100),
and 537 (34). (Abundance relative to m/e 547).

Found: C, 56.6; H, 6.31. $\text{C}_{33}\text{H}_{42}\text{O}_{17}$ requires C, 55.8; H, 5.9%.

Hydrolysis of Detigloyl-pyruvyl-dihydro-azadirachtin

A crude sample of detigloyl-pyruvyl-dihydro-azadirachtin
(42 mg.) was dissolved in methanol and saturated sodium bicarbonate solution
was added. After ten minutes the solution was extracted with chloroform,
the chloroform was washed with water and evaporated leaving a colourless
residue (34 mg.) which on t.l.c. in ether-acetone (4:1) showed the major
component to be starting material R_F 0.19, a spot at R_F 0.26 corresponding
to the impurity in the starting material and a new component R_F 0.10.

The residue was treated under the same conditions for a further
hour, the material obtained on evaporation of the chloroform extract showed
on t.l.c. in ether-acetone (4:1) the new component R_F 0.10 as the major
spot with the minor components corresponding to starting material and
starting material impurity. The n.m.r. spectrum of the residue showed
that the absorption at $\tau 7.5$ was considerably less intense than in the
spectrum of detigloyl-pyruvyl-dihydro-azadirachtin (fig. 10).

The hydrolysis mixture was subjected to P.L.C. eluting twice with 4:1 ether-acetone. *Detigloyl-dihydro-azadirachtin* was isolated as a colourless glassy solid (14 mg.) m.p. 164-168° R_F Ether-acetone (4:1) 0.10, ethyl acetate 0.16 v_{max} . ($CHCl_3$) 3440 (broad), 1740 (strong), 1720 cm^{-1} (shoulder). Found: C, 55.7; H, 6.3%. $C_{30}H_{40}O_{15}$ requires C, 56.3; H, 6.3%. The n.m.r. spectrum is shown in fig. 11. The mass spectrum showed significant peaks above m/e 400 at 622 (2), 604 (2.5), 590 (4.5), 579 (9), 562 (14), 547 (7), 537 (4.5), 519 (22), 505 (9), 477 (45), 467 (14), 451 (9), 439 (100). (Abundance relative to m/e 439).

Attempted Oxidation of Detigloyl-dihydro-azadirachtin with
Cornforth's Reagent (Chromium Trioxide-Pyridine)

Cornforth's Reagent was prepared by adding a solution of chromium trioxide (5 g.) in water (3 ml.) to pyridine (50 ml.) cooled in ice.

A crude sample of detigloyl-dihydro-azadirachtin (77 mg.) was dissolved in pyridine (0.25 ml.), Cornforth's reagent (2.0 ml.) was added and the mixture was left for 65 hours at room temperature. Water (15 ml.) was then added and the solution was extracted with chloroform, the chloroform was washed with water and evaporated leaving a brown residue (66 mg.) which on t.l.c. in ether-acetone (4:1) showed components at the origin, R_F 0.13 and R_F 0.22.

The residue was subjected to P.L.C. eluting twice with ether-acetone (85:15). Two bands were visible under ultraviolet light. The

upper band gave a colourless residue (15 mg.) and the lower band a colourless residue (17 mg.).

The residue from the lower band on t.l.c. in ether-acetone (4:1) showed a single spot R_F 0.10 corresponding to detigloyl-dihydro-azadirachtin, the n.m.r. spectrum of this fraction was identical with that of the starting material.

The residue from the upper band on t.l.c. in ether-acetone (4:1) showed a single spot at R_F 0.18, however on t.l.c. in ethyl acetate two spots were visible a major one R_F 0.34 and a minor one R_F 0.26. This residue was subjected to P.L.C. eluting once with ethyl acetate, the major component, an $\alpha\beta$ -unsaturated ketone was isolated as a slightly yellow glassy material (9.5 mg.) m.p. 145-148° R_F ethyl acetate 0.35. ν_{\max} . 3500 (broad), 1740 (strong), 1690 cm^{-1} (medium). λ_{\max} . 225 nm. $E_1^{1\%}$ cm. 2300. The accumulated n.m.r. spectrum is shown in fig. 12. The mass spectrum was uninformative showing ions of higher mass than the starting material.

The silica between the origin and the major band was worked up in the usual way to give a glassy material (6 mg.) which on t.l.c. in ethyl acetate showed a streak from the origin, a major spot R_F 0.25 and a faint spot at R_F 0.35. The n.m.r. spectrum of this material showed broad ill-defined peaks not characteristic of a single compound.

Attempted Reduction of Azadirachtin with Sodium Borohydride

i) The ultraviolet spectrum of azadirachtin was recorded at a concentration of 125 mg./l. in 95% ethanol. A small amount of sodium borohydride was added to both the sample and reference cell and the spectrum was recorded, several times over a period of 30 minutes, no significant change in the intensity of the absorption at 217 nm. was observed.

ii) Azadirachtin (11 mg.) was dissolved in dioxan-water (4:1, 4 ml.), sodium borohydride (4 mg.) was added and the mixture was left at room temperature for 20 minutes. Water was then added and the mixture was extracted with chloroform, the chloroform was washed with water and evaporated. The residue on t.l.c. in chloroform-acetone (7:3) showed only azadirachtin R_F 0.4.

The recovered azadirachtin was treated with sodium borohydride under the same conditons, the mixture was left overnight and then worked up as before. The residue on t.l.c. in chloroform-acetone (7:3) showed a streak with no clearly defined spots from the origin to R_F 0.45.

Attempted Lactone Titration of Azadirachtin

Azadirachtin (59 mg.) was dissolved in ethanol (20 ml.) and approximately 0.01M sodium hydroxide solution (20 ml.) was added. The solution was immediately titrated with 0.01M hydrochloric acid using

phenolphthalein as indicator. A blank titration was carried out on ethanol (20 ml.) and 0.01M sodium hydroxide (20 ml.). From the difference in titres it was calculated that 0.53 equivalents of base had been consumed. The solution was acidified with dilute hydrochloric acid and extracted with chloroform, the chloroform was washed with water and evaporated. The residue on t.l.c. in ether-acetone (4:1) showed two components, a major one at R_F 0.51 and a minor one at R_F 0.38. The residue was subjected to P.L.C. eluting twice with ether-acetone (95:5), the major band gave a colourless residue (16 mg.) m.p. 114-118° R_F ether-acetone (4:1) 0.55. ν_{\max} . 3460 (broad), 1740 (strong), 1720 (strong), 1650 (weak) and 1620 cm^{-1} (weak). The accumulated n.m.r. spectrum of this residue was very similar to that of azadirachtin except that the high field methoxyl absorption was very much reduced in intensity and there was an increase in the intensity of absorption at τ 8.7.

The mass spectrum showed the following ions above m/e 600, 716 (2.5), 702 (10), 688 (15), 684 (12), 670 (12), 656 (15), 643 (30), 635 (100) and 627 (18) (Abundance relative to m/e 635).

Alkaline Hydrolysis of Azadirachtin

i) Azadirachtin (100 mg.) was dissolved in methanol (6 ml.) and 5% aqueous potassium hydroxide solution was added, the solution immediately became yellow. After two hours the solution was acidified and extracted with ethyl acetate. The ethyl acetate extract was washed with water and

evaporated leaving a yellow residue (57 mg.) which was insoluble in chloroform, on t.l.c. in ether-acetone (4:1) it showed an elongated spot at the origin with some streaking upwards. The residue was dissolved in methanol and treated with ethereal diazomethane. Evaporation of the solvent gave a yellow residue which on t.l.c. in ether-acetone (4:1) showed major components at R_F 0.23 and R_F 0.32 with minor components at R_F 0.40, R_F 0.46 and R_F 0.54. The n.m.r. spectrum of this residue showed broad ill-defined absorptions in the τ 6.1- τ 6.4 and τ 7.7- τ 8.4 regions with an intense absorption at τ 8.7.

The residue was subjected to P.L.C. eluting twice with ether-acetone (9:1), five components were isolated. The accumulated n.m.r. spectra of these components were ill-defined and no conclusions could be made from them.

ii) Azadirachtin (200 mg.) was dissolved in methanol (10 ml.) and 2½% aqueous potassium hydroxide solution (10 ml.) was added. After one hour the solution was made slightly acid with dilute hydrochloric acid, extracted with ethyl acetate in the normal way and then continuously extracted with ethyl acetate for two hours.

The ethyl acetate was evaporated leaving a yellow residue (160 mg.). Chloroform (~ 1 ml.) was added to the residue, most of which appeared to remain undissolved. The mixture was cooled to 0°C and treated with ethereal diazomethane, after 20 minutes the solvent was evaporated leaving a yellow glassy residue which on t.l.c. in ether-acetone (4:1) showed a major component at R_F 0.32 and two minor components at R_F 0.37 and R_F 0.45. The n.m.r. spectrum of the residue was very similar to that

of the crude product obtained in the previous experiment except that the $\tau 8.7$ absorption was much less intense.

The residue was subjected to P.L.C. eluting twice with ether-acetone (95:5), the major component, *deacetyl-azadirachtin* was isolated as a colourless glass (25 mg.) m.p. $159-164^{\circ}$ R_F ether-acetone (4:1) 0.32.

Found: C, 57.9; H, 6.7% $C_{33}H_{42}O_{15}$ requires C, 58.4; H, 6.2%.

ν_{\max} . ($CHCl_3$) 3440 (broad), 1740 (strong), 1655 (weak), 1630 cm^{-1} (weak).

The n.m.r. spectrum is shown in fig. 13. The mass spectrum showed the following ions above m/e 500 at 660 (8), 646 (7), 642 (7), 633 (18), 628 (14), 612 (12), 600 (13), 585 (19), 551 (72), 528 (27), 519 (100). (Abundance relative to m/e 519).

The band corresponding to the R_F 0.37 component gave a colourless residue (14 mg.), which on t.l.c. in ether-acetone (4:1) showed the main component at R_F 0.37, with some material R_F 0.32. The n.m.r. spectrum showed complex absorptions at $\tau 6.2$ and between $\tau 7.8-8.3$ and a strong absorption at $\tau 8.7$.

Alkaline Hydrolysis of Dihydro-azadirachtin

Dihydro-azadirachtin (300 mg.) was dissolved in methanol (15 ml.) and 2½% potassium hydroxide solution (15 ml.) was added. After one hour the solution was acidified with dilute hydrochloric acid and extracted with ethyl acetate continuously for one hour. The ethyl acetate was washed with water and evaporated leaving a yellow residue, which appeared

to be insoluble in chloroform. Chloroform and ethereal diazomethane was added to the residue, after ten minutes at 0°C the solvent was evaporated leaving a yellow glassy residue (257 mg.), which on t.l.c. in ether-acetone (4:1) showed two major components R_F 0.20 and R_F 0.30.

The residue was subjected to P.L.C. eluting twice with ether-acetone (9:1). The less polar component was isolated as a colourless solid (60 mg.) soluble in chloroform, R_F 0.30 in ether-acetone (4:1). The n.m.r. spectrum is shown in fig. 15. The mass spectrum showed ions above 600 at 704 (25), 690 (22), 679 (93), 672 (15), 649 (90), 635 (36), 629 (100), 619 (40), 605 (70) (Abundance relative to m/e 629).

ν_{\max} . (CHCl_3) 3460 (broad), 1730 (strong) 1650 cm^{-1} (weak).

This product appeared to change in character, after evaporation of the solvents it became insoluble in chloroform.

The other component *deacetyl-dihydro-azadirachtin* was isolated as a colourless glassy solid (48 mg.) R_F 0.20 in ether-acetone (4:1) m.p. 137-142° ν_{\max} . 3460 (broad), 1740 (strong) and 1655 cm^{-1} (weak). The n.m.r. spectrum is shown in fig. 14. The mass spectrum showed ions above m/e 500 at 662 (5), 648 (5), 644 (7), 630 (23), 621 (7), 616 (7), 602 (38), 598 (13), 587 (30), 559 (63), 545 (50), 531 (25), 517 (100) (Abundance relative to m/e 517).

Treatment of Azadirachtin with Acid

i) Azadirachtin (68 mg.) was dissolved in 2N. methanolic

hydrochloric acid (10 ml. prepared from conc. hydrochloric acid and methanol) after two hours at room temperature water was added and the aqueous solution was extracted with chloroform, the chloroform was washed with water, dried (MgSO_4), filtered and evaporated leaving a colourless residue (50 mg.) which on t.l.c. in ether-acetone (4:1) showed the major component at R_F 0.36 along with three minor components. The n.m.r. spectrum showed that the two doublets present in the spectrum of azadirachtin at τ 3.65 and τ 3.95 were no longer apparent, there was also considerable change in the τ 6.2-6.3 region with the appearance of an intense peak at τ 6.6. The region from τ 8.0-8.4 was also changed.

The residue was subjected to P.L.C. eluting once with ether-acetone (9:1), the major component was isolated as a colourless residue (16 mg.) which on t.l.c. in ether-acetone (4:1) appeared to consist of two or three components very close together at R_F 0.35. The n.m.r. spectrum was similar to that of the crude mixture which had been chromatographed. The infrared spectrum did not show the absorption at 1625 cm^{-1} present in the spectrum of azadirachtin. The relative intensity of the hydroxyl absorption at 3460 cm^{-1} to the carbonyl absorption was the same as that in the spectrum of azadirachtin, indicating that no hydrolysis of the ester groups had taken place.

Treatment of azadirachtin with 0.1N methanolic hydrochloric acid at -5° for three days gave a similar reaction product.

Treatment of Azadirachtin with Aqueous Acid

Azadirachtin (50 mg.) was dissolved in 0.5N aqueous ethanolic hydrochloric acid (30 ml. equal volumes ethanol and 1N aqueous hydrochloric acid). After 15 minutes the solution was extracted with chloroform, the chloroform was washed with water and evaporated. The n.m.r. spectrum of the residue was identical to that of azadirachtin.

Treatment of Dihydro-azadirachtin with Acid

Dihydro-azadirachtin (~ 1 mg.) was dissolved in 2N methanolic hydrochloric acid (0.5 ml. prepared from conc. hydrochloric acid and methanol). After one hour the solvent was evaporated leaving a residue which on t.l.c. in ether-acetone (4:1) showed components R_F 0.02, R_F 0.11, R_F 0.26 (dihydro-azadirachtin) and R_F 0.40.

Action of Dry Hydrogen Chloride on Azadirachtin

Azadirachtin (39 mg.) was dissolved in chloroform (0.5 ml.) and a solution of chloroform saturated with hydrogen chloride (0.5 ml.) was added. After two minutes the solution on t.l.c. in ether-acetone (9:1) showed components at R_F 0.3 and R_F 0.25 of equal intensity with minor components at R_F 0.1, R_F 0.44 and R_F 0.59. After five and 25 minutes

no further change was observed.

The solvent was evaporated and the colourless residue was subjected to t.l.c. in ether-acetone (9:1) this showed the major spot at R_F 0.25 which appeared to be two components, an elongated spot at the origin and a faint spot R_F 0.55. The residue was subjected to P.L.C. eluting with ether-acetone (9:1) only one major band was observed under ultraviolet light, extraction gave a colourless solid m.p. 125-128° ν_{\max} . (CCl_4) 3450 (broad), 1740 (strong), 1720 (shoulder) and 1710 cm^{-1} (shoulder). The accumulated n.m.r. spectrum was very similar to that of azadirachtin except that the doublets at τ 3.65 and τ 4.95 were no longer present.

Bromination of Azadirachtin

- i) A solution of bromine in chloroform was added to azadirachtin (1 mg.) dissolved in chloroform. After a few minutes the solution was evaporated leaving a residue which on t.l.c. in ether-acetone (49:1) showed the major component R_F 0.07 with minor components at R_F 0, R_F 0.14 (azadirachtin) and R_F 0.22.
- ii) A small amount of calcium carbonate was suspended in a solution of azadirachtin (38 mg.) in chloroform. A solution of bromine in chloroform was added drop by drop until the bromine colour persisted, the solution was then filtered and evaporated. The residue on t.l.c. in ether-acetone (4:1) showed the major component at R_F 0.64 with some azadirachtin at R_F 0.52. The residue was subjected to P.L.C. eluting

with ether-acetone (95:5) the major component was isolated as a colourless glassy residue (19 mg.) m.p. 139-142°.

$\nu_{\text{max.}}$ 3460 (broad), 1745 (strong), 1715 (strong), 1655 cm^{-1} (weak)

$\lambda_{\text{max.}}$ 219 nm. $\epsilon_{\text{max.}}$ 7000 (assuming mol. wt. 880). The n.m.r. spectrum is shown in fig. 16.

Lithium Aluminium Hydride Reduction of Azadirachtin

Azadirachtin (40 mg.) was dissolved in tetrahydrofuran (0.5 ml.) (dried by refluxing over lithium aluminium hydride and then distilling) and a solution of lithium aluminium hydride (10 mg.) in tetrahydrofuran (1.5 ml.) was added. The mixture was shaken for two hours at room temperature and then a solution of water in tetrahydrofuran was added, there was immediate effervescence. The mixture was centrifuged and the cloudy solution was pipetted off, the precipitate was extracted with more tetrahydrofuran and the combined extracts were evaporated to dryness leaving a colourless residue which was insoluble in chloroform, carbon tetrachloride, acetone and benzene, but was partially soluble in 95% ethanol. Ethanol was added to the residue the ethanol solution was filtered and evaporated leaving a colourless residue (8 mg.) which on t.l.c. in 95% ethanol showed three spots with considerable streaking between them at R_F 0.20, R_F 0.46 and R_F 0.63. The infrared spectrum showed a broad intense absorption at 3400 cm^{-1} due to hydroxyl and also a weak absorption at 1720 cm^{-1} indicating the presence of carbonyl.

Acetylation of Azadirachtin

i) Acetic Anhydride

a) Azadirachtin (6 mg.) was warmed with acetic anhydride (0.5 ml.) on a steam bath for 30 minutes. Water was then added and the aqueous solution was extracted twice with benzene. The benzene was washed with water, dried (MgSO_4), filtered and evaporated leaving a light brown residue which on t.l.c. in ether acetone (49:1) showed a weak component R_F 0.20, an intense component R_F 0.14 corresponding to azadirachtin and a weak component R_F 0.05.

b) Azadirachtin (100 mg.) was heated under reflux with acetic anhydride (10 ml.) for ten minutes, then the acetic anhydride was evaporated leaving a light brown residue which on t.l.c. in ether-acetone (4:1) showed a spot R_F 0.50 corresponding to azadirachtin and a spot R_F 0.40. The residue was subjected to P.L.C. eluting twice with ether-acetone (9:1). The upper band gave azadirachtin (24 mg.) and the lower band gave *acetyl-azadirachtin* (35 mg.) m.p. 154-156° R_F 0.40 in ether-acetone (4:1).

Found: C, 58.2; H, 6.45, $\text{C}_{37}\text{H}_{46}\text{O}_{17}$ requires C, 58.2; H, 6.04%.

ν_{max} . (CCl_4) 3450 (broad), 1750 (strong), 1715 (strong), 1655 (weak) and 1625 cm^{-1} (weak). The n.m.r. spectrum at 60 MHz. and 100 MHz. is shown in figs. 17 and 18. The mass spectrum showed ions above m/e 500 at 744 (1.3), 702 (12), 685 (56), 670 (11), 661 (25), 643 (44), 625 (44), 602 (100), 559 (66). (Abundance relative to m/e 602).

ii) Pyridine-Acetic Anhydride

Azadirachtin (8 mg.) was dissolved in pyridine (0.2 ml.) and acetic anhydride (0.2 ml.). After two days at room temperature, the pyridine and acetic anhydride were evaporated (0.1 mmHg.) leaving a light brown residue which was identified as azadirachtin by t.l.c.

iii) Pyridine-Toluene-Acetic Anhydride

To a solution of azadirachtin (2 mg.) in toluene (1.5 ml.) was added pyridine (20 drops) and acetic anhydride (20 drops) and the mixture was refluxed. After 30 minutes a small volume of the solution was removed and evaporated (0.1 mmHg.) leaving a residue which on t.l.c. in ether-acetone (49:1) showed two main components R_F 0.08 and R_F 0.16 (Azadirachtin) with very minor components R_F 0.38, R_F 0.48 and R_F 0.9.

After four hours the remainder of the material was worked up similarly, the residue on t.l.c. showed the main spot R_F 0.06 with minor spots R_F 0.16 (Azadirachtin), R_F 0.45 and R_F 0.9.

iv) Acetic Anhydride-Boron Trifluoride Etherate

a) A mixture of acetic anhydride (8 ml.) and boron trifluoride etherate (2 drops) was prepared. A portion of this mixture (1.5 ml.) was added to azadirachtin (5 mg.), after a few minutes the solution became

noticeably yellow. After two hours the solution was evaporated (0.1 mmHg.) leaving a brown residue, which on t.l.c. in ether-acetone (49:1) remained at the origin.

b) Similarly a mixture of acetic anhydride (25 ml.) and boron trifluoride etherate (1 drop) was allowed to stand in contact with azadirachtin for 100 minutes the residue on t.l.c. showed a mixture of five components, treatment for a longer time did not produce any further change.

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